EFFECTS OF TELAZOL®, TILETAMINE, AND ZOLAZEPAM ON HEPATIC CYTOCHROME P450 EXPRESSION IN RATS

by

ANNE WONG

B. Sc. (Pharm.), The University of British Columbia, 1993

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

FACULTY OF PHARMACEUTICAL SCIENCES

Division of Pharmaceutical Chemistry

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September 1996

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Department of Pharmaceutical Sci.

The University of British Columbia
Vancouver, Canada

Date Sept 25/86

ABSTRACT

The goal of the present project was to examine the effects the immobilizing agent, Telazol®, and its constituents, tiletamine and zolazepam, on cytochrome P450 expression in rats. The effects elicited by the three agents were compared with those produced by phenobarbital (PB) using a similar dosing regimen.

In the first of three sections, male Long Evans rats received a single i.p. injection of Telazol® at a dose of 20, 40, 80, or 120 mg/kg. Rats were killed 24 h later and hepatic microsomes prepared. Treatment with Telazol® produced a dose-dependent increase in benzyloxyresorufin (BROD) and pentoxyresorufin (PROD) *O*-dealkylase, and testosterone 16β-hydroxylase activities. The same treatment group showed a significant increase of 17-fold in the hepatic level of cytochrome P450 2B1. A dose-dependent but insignificant increase was found in the hepatic level of cytochrome P450 2B2.

In section 2, male Long Evans rats received a single i.p. injection of tiletamine at a dose of 60 mg/kg, zolazepam at a dose of 60 mg/kg, or Telazol® at a dose of 120 mg/kg. Rats were killed 24 h later and hepatic microsomes prepared. Tiletamine produced a small but insignificant increase in BROD and testosterone 16β-hydroxylase activities, whereas zolazepam and Telazol® increased the enzyme activities by 4.7- to 15.3-fold, respectively. The lather treatments also significantly increased hepatic levels of cytochrome P450 2B1, P450 2B2, and P450 3A isozymes.

In section 3, male Long Evans rats received a single i.p. injection of PB at a dose of 60 or 120 mg/kg. Rats were killed 24 h later and hepatic microsomes prepared. PB at a dose of

120 mg/kg increased BROD, EROD, and PROD activities by 22.6-, 2.0-, and 7.0-fold, respectively, while significant increases of 1.7-, 17.4-, and 1.7-fold, respectively, were found in the rates of formation of 6β-, 16β-hydroxytestosterone, and androstenedione. The same treatment elevated hepatic levels of cytochrome P450 2B and P450 3A isozymes by 24.9- and 8.9-fold, respectively. Treatment with PB at a dose of 60 mg/kg produced similar increases in enzyme activities and protein contents of cytochromes P450.

In summary, results indicated that Telazol®, at a dose of 80 mg/kg or greater, induced hepatic expression of cytochrome P450 2B in rats. Zolazepam was the major constituent responsible for the enzyme induction. The pattern of induction produced by Telazol® was similar to that elicited by PB, although PB at a similar dose produced a greater inductive effect on cytochromes P450.

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LIST OF ABBREVIATIONS

AhR	aromatic hydrocarbon receptor
AhRE	aromatic hydrocarbon-responsive elements
ANOVA	analysis of variance
βNF	β-naphthoflavone
BIS	N,N'-methylene-bis-acrylamide
BSA	bovine serum albumin
BROD	benzyloxyresorufin *O*-dealkylase
CYP	cytochrome P450
DDT	dichloro-diphenyl-trichloroethane
DEX	dexamethasone
EDTA	ethylenediaminetetraacetic acid
EROD	ethoxyresorufin *O*-deethylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
hsp 90	heat shock protein
IgG	immunoglobulin
i.p.	intraperitoneally
3-MC	3-methylcholanthrene
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	*p*-nitroblue tetrazolium chloride

NMDA	*N*-methyl-D-aspartate
PB	phenobarbital
PBRE	phenobarbital-responsive elements
PCBs	polychlorinated biphenyls
PCN	pregnenolone-16α-carbonitrile
PROD	pentoxyresorufin *O*-depentylase
PPAR	peroxisome proliferator activated receptor
SDS	sodium dodecyl sulphate
TAO	triacetyloleandomycin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEMED	N,N,N',N'-tetramethyethylenediamine

ACKNOWLEDGEMENTS

I would like to give a special thanks to my supervisor, Dr. S. Bandiera, for all his support and guidance for the past two years. I would like to thank my committee members, Dr. F. Abbott, Dr. H. Burt, Dr. M. Levine, and Dr. K. McErlane for their valuable advice. I would like to thank my parents for their continuing love and understanding regardless of how little time I spend with them. And I would like to thank my friends who have been with me throughout all these years.

1. INTRODUCTION

The aim of my thesis research was to determine the effects of the immobilizing agent, Telazol®, and its constituents, tiletamine and zolazepam, on hepatic cytochromes P450, using rat as the experimental model. Telazol® has been used extensively as an anesthetic and immobilizing agent in various domestic and wild animal species such as cats, dogs, leopards, lions and polar bears (1). Recently, Telazol® was used in a study that examined the measurement of hepatic cytochrome P450 levels in polar bears as a biomarker of exposure to environmental contamination in the Arctic ecosystem (2, 3). That study was based on the previous observation that animal exposure to environmental contaminants was found to accompany induction of hepatic cytochromes P450 (2, 3). Hence, an effort was made to correlate contaminant concentrations found in polar bear fat tissues with expression of specific cytochrome P450 isozymes, namely cytochrome P450 1A and cytochrome P450 2B. Before a correlation could be established between the two parameters, however, it was necessary to determine whether Telazol® , which had been given to polar bears 0.5 to 11 days prior to death, had any effect on hepatic cytochrome P450 expression.

The cytochrome P450 system has been studied extensively for almost 30 years. Cytochrome P450 was discovered initially by its carbon-monoxide binding pigment which absorbs light maximally at 450 nm (4). The pigment was later determined to be a heme-containing cytochrome (4) and found to function as a terminal oxidase in an electron transport pathway (5). Cytochromes P450 are present in both eukaryotes and prokaryotes including bacteria, plants, insects, fish and mammals (6). The enzymes catalyze the biotransformation of

lipophilic endogenous compounds, such as cholesterol, steroids and fatty acids, and xenobiotic compounds including various drugs and carcinogens, into more polar metabolites to facilitate their subsequent excretion. Multiple forms of cytochromes P450 have been purified over the years. Each isozyme possesses unique, but partially overlapping substrate specificity.

Several cytochrome P450 isozymes are subject to induction by a wide variety of compounds such as 3-methylcholanthrene (3-MC) (7), barbiturates (7, 8), and environmental contaminants (9, 10). As a result of induction , drug biotransformation is enhanced possibly leading to increased production of toxic drug intermediates, decreased pharmacological activities, or drug-drug interactions. In this study, we were interested in determining whether treatment with Telazol®, tiletamine, or zolazepam alters hepatic expression of cytochromes P450. At present, there is no information in the literature regarding the effect of Telazol®, or its constituents, on cytochromes P450.

By way of background, I will present a brief introduction on the catalytic properties of cytochromes P450, and describe various cytochrome P450 inducers and their proposed mechanisms of induction. In addition, the pharmacological activities of Telazol® and its constituents will be discussed.

1.1. *CATALYTIC CYCLE OF CYTOCHROME P450-DEPENDENT MONOOXYGENASE REACTION*

Cytochrome P450 is the terminal enzyme of an electron transport chain (5). It is a hemeprotein consisting of an iron protoprophyrin IX heme moiety and a single polypeptide chain or apoprotein of 45,000 to 55,000 Da (11). The iron of the heme prosthetic group is located at the center of the protoporphyrin ring. Four ligands from the heme iron are coordinated to the porphyrin ring (12). The fifth ligand is a thiolate anion from a cysteinyl residue of the apoprotein (12), and the sixth ligand is the site where binding and activation of molecular oxygen occurs (13).

In monooxygenase reactions, cytochromes P450 receive electrons from an iron-sulfur protein such as adrenodoxin (14) or putidaredoxin (15), or from the flavoprotein, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cytochrome P450 reductase (16). In mammalian cells, the iron-sulfur-dependent enzymes are located in the mitochondria and are involved in the biosynthesis of endogenous compounds such as steroid hormones and bile acids, as well as in the metabolism of vitamin D_3 (6). The hepatic enzymes, on the other hand, are supported by NADPH-cytochrome P450 reductase and are located primarily in the endoplasmic reticulum (17, 18). The hepatic microsomal cytochromes P450 are involved mainly in the metabolism of xenobiotic compounds. Cytochromes P450 catalyze a wide variety of monooxygenase reactions, including hydroxylations, epoxidations, *N*-demethylations, sulfoxidations and oxidative dehalogenations (12). A typical reaction cycle of cytochrome P450 is represented in Figure 1.1.

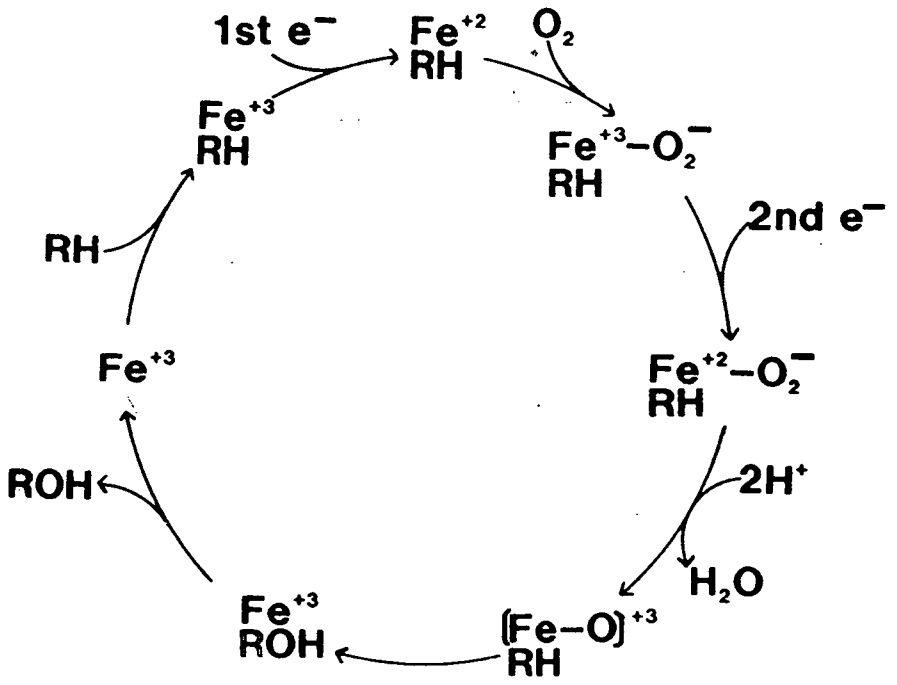


Figure 1.1. Schematic diagram of cytochrome P450 monooxygenase reaction. RH represents a substrate and ROH the product. Adapted from Omura *et al.*, 1993. (19).

Oxidation of compounds via the cytochrome P450 system is initiated by binding of a substrate to an oxidized cytochrome P450, which is reduced by the flavoprotein, cytochrome P450 reductase, in an NADPH-dependent reaction. Electrons from NADPH are transferred to cytochrome P450 and used to reduce molecular oxygen to generate an active oxygen species, which is subsequently inserted into the substrate. Cleavage of the dioxygen bond yields a hydroxylated product that is more hydrophilic than the original compound. The product in turn may undergo further biotransformation by Phase I or Phase II enzymes before being excreted from the body.

1.2 *GENE NOMENCLATURE OF CYTOCHROMES P450*

Numerous cytochrome P450 forms have been identified since the purification of the first cytochrome P450 in the early 1970's. With advances in molecular biology techniques in recent years, many fundamental features of various cytochrome P450 genes have been characterized, which have been helpful in the classification of the cytochrome P450 gene superfamily. A systematic nomenclature based on the similarity of amino acid sequences has been proposed and is currently in use (20). In this nomenclature, a cytochrome P450-protein sequence from one gene family is defined as having $\leq 40\%$ identity with that from any other family (20), and cytochrome P-450 genes that share a $\geq 55\%$ sequence homology are grouped in the same subfamily (20). Each cytochrome P450 isozyme is identified by a three character code. The family of cytochromes P450 is designated by an Arabic number, the subfamily is indicated by a capital letter, and the indvidual gene is described by an Arabic numeral (20). Thus far, there are 36 gene families, including 12 mammalian gene families and 22 mammalian subfamilies (20). A table summarizing various genes of rat cytochromes P450 and their corresponding trivial names is provided below (Table 1.1).

1.3. *CYTOCHROME P450 SUPERFAMILY*

Among the mammalian gene families examined to date, the cytochrome P450 1, cytochrome P450 2 and cytochrome P450 3 gene families play the most important role in the metabolism of xenobiotic compounds, while the cytochrome P450 4 gene family is involved mainly in the metabolism of endogenous compounds such as fatty acids. In the following

sections, several subfamilies of these four major gene families will be described in terms of their amino acid sequence similarity, catalytic properties and inducibility.

Table 1.1. Nomenclature of major cytochromes P450 (CYP) in rats. Adapted from Nelson *et al.*,1993 (20).

Gene symbol	Trivial name
CYP1A subfamily	
CYP1A1	c, βNF-B
CYP1A2	P-448, d, HCB
CYP2A subfamily	
CYP2A1	a1, a, 3, UT-F, RLM2b, IF-3
CYP2A2	a2, RLM2, UT-4
CYP2B subfamily	
CYP2B1	b, PB-4, PB-B, PBRLM5
CYP2B2	3, PB-5, PB-D, PBRLM6
CYP2B3	IIB3
CYP2C subfamily	
CYP2C6	PB1, k, PB-C, pTF2, RLM5a
CYP2C7	f, RLM5b, pTF1
CYP2C11	h, M-1, 16α, 2c, UT-A, RLM5, male
CYP2C12	i, 15β, 2d, UT-1, female
CYP2C13	+g, -g, RLM3, UT-5
CYP2D subfamily	
CYP 2D1	UT-7, db1, CMF1a
CYP 2D2	db2, CMF2
CYP2E subfamily	
CYP2E1	j, RLM6
CYP3A subfamily	
CYP3A1	p, pcn1, PCNa, 6β-4
CYP3A2	l, pcn2, PCNb/c, PB-1, 6β-1/3
CYP4A subfamily	
CYP4A1	LAω

1.3.1 *Cytochrome P450 1A subfamily*

In mammals, the cytochrome P450 1A subfamily consists of two genes, cytochrome P450 1A1 and cytochrome P450 1A2. These forms were previously termed cytochrome P448 because both absorb light maximally at 448 nm as determined by reduced-carbon monoxide difference spectra (21). The two isozymes share 68% amino acid sequence similarity (22, 23). Cytochrome P450 1A1 is expressed in rat liver at very low levels normally, but is highly inducible upon exposure of the animal to lipophilic planar arenes such as 3-MC, β-naphthoflavone (βNF), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), referred to collectively as 3-MC-type inducers (24). This cytochrome P450 isozyme has high catalytic activity for benzo[a]pyrene hydroxylation (25, 26). Cytochrome P450 1A2, on the other hand, is constitutively expressed at a higher level, and unlike cytochrome P450 1A1, its expression is largely restricted to the liver (27). Cytochrome P450 1A2 can be induced by 3-MC-type inducers and exhibits high catalytic activity toward arylamines (28, 29). Both forms of cytochrome P450 have overlapping substrate specificities and catalyze reactions such as ethoxyresorufin *O*-deethylation (EROD) (27). The cytochrome P450 1A family is thought to play an important role in activation of various carcinogens.

1.3.2. *Cytochrome P450 2A subfamily*

Two cytochrome P450 2A proteins, cytochrome P450 2A1 and cytochrome P450 2A2 have been isolated in rat liver. Their amino acid sequences are 88% similar (30). Cytochrome P450 2A1 and cytochrome P450 2A2 catalyze the regioselective hydroxylation of testosterone predominantly at the 7α and 15α positions, respectively (30). Expression of the two

cytochrome P450 genes is regulated differently. Production of cytochrome P450 2A1 is increased in immature male and female rats, but is suppressed in males at the onset of puberty (31, 32). Cytochrome P450 2A2, on the other hand, is not expressed in females but is expressed in males at puberty (33). The sexually differentiated expression of cytochrome P450 2A enzymes is thought to be controlled partly by the pattern of pituitary growth hormone secretion (31). Cytochrome P450 2A1 can be induced by treatment with 3-MC, while cytochrome P450 2A2 is refractory to induction (27). A third related gene, cytochrome P450 2A3, is found in rat lung and can also be induced by 3-MC (34).

1.3.3. *Cytochrome P450 2B subfamily*

Two members of this subfamily, cytochrome P450 2B1 and cytochrome P450 2B2, have been studied extensively due to their inductive response to phenobarbital (PB). Because the isozymes share 97% amino acid sequence homology (35, 36), they are immunochemically crossreactive, although they can be distinguished on immunoblots by virtue of their slightly different mass to charge ratio. Both cytochrome P450 2B1 and cytochrome P450 2B2 are minor constituents of total cytochrome P450 in untreated rat liver (37). Cytochrome P450 2B1 protein is present at a level at least 5- to 10-fold lower than that of cytochrome P450 2B2 (37). Upon exposure of animals to PB or PB-like inducers, such as dichloro-diphenyl-trichloroethane (DDT) however, both forms are elevated significantly above basal levels (20 to > 100-fold increase) with expression of cytochrome 2B1 induced to a greater extent than cytochrome P450 2B2 (38, 39). Reactions that are catalyzed by cytochrome P450 2B isozymes include 7-pentoxyresorufin *O*-dependentylation (PROD) (40, 41), benzyloxyresorufin *O*-dealkyation

(BROD) (41), benzphetamine *N*-demethylation (42, 43), and testosterone 16β-hydroxylation (43). A non-inducible form of this subfamily, cytochrome P450 2B3, has also been found to be expressed constitutively in liver of male and female rats (44). Cytochrome P450 2B3 is 77% similar to cytochrome P450 2B1 and cytochrome P450 2B2 at the amino acid level (44), and thus is immunochemically crossreactive with the two isozymes. The biological role of cytochrome P450 2B3 is presently unclear. Another isoform, cytochrome P450 2B8 has also been identified in rat liver (45). The hepatic level of cytochrome P450 2B8 is very low in untreated animals, but transcription of this gene can be increased by approximately 6-fold upon treatment with PB (45).

1.3.4. *Cytochrome P450 2C subfamily*

The cytochrome P450 2C subfamily is a class of constitutively expressed enzymes. The amino acid sequence similarities between members of cytochrome P450 2C subfamily range from 68 to 75% (27). Regulation of cytochrome P450 2C isozymes may differ depending on the age, gender and strain of the animals. Cytochrome P450 2C6 is expressed constitutively in liver of male and female rats (46). It is inducible by treatment with PB and a two- to four-fold increase in enzyme expression is seen following drug treatment (47). Cytochrome P450 2C6 is regulated developmentally, with significant levels being present only 3 weeks after birth (46). The regulation of another isozyme, cytochrome P450 2C7, is also developmentally-dependent. Expression of cytochrome P450 2C7 increases from less than 1% of total cytochrome P450 in immature rats to 7% and 14% of total cytochrome P450 in adult male and female rats, respectively (43). Both cytochrome P450 2C6 and cytochrome P450 2C7 are involved in the

metabolism of progesterone (43). Cytochrome P450 2C11 and cytochrome P450 2C13 are male-specific isozymes that are regulated developmentally and are refractory to induction. Cytochrome P450 2C11 catalyzes the hydroxylation of testosterone at the 2α, 16α, and C17 positions, whereas cytochrome P450 2C13 mediates hydroxylation at the 6β and 15α positions (43). Regulation of cytochrome P450 2C11 and cytochrome P450 2C12, a female-specific isozyme, appears to be dependent on the pattern of growth hormone secretion (48, 49, 50). Cytochrome P450 2C11 is non-detectable in newborn male rats, but hepatic levels of this isozyme increase at puberty and decline at senility (older than 25 months) (51). Expression of cytochrome P450 2C11 is dependent on testosterone and thus does not occur in female rats (31, 49, 50, 51). Expression of cytochrome P450 2C11 is often suppressed by inducers such as 3-MC and PB (52, 53). Cytochrome P450 2C12, on the other hand, is absent in neonatal female rats, develops at 2 to 4 weeks of age and reaches maximal levels in adulthood (51, 54). This isozyme is also found at low levels in immature male and very old male rats (51), and is mainly involved in the oxidation of androstanediol disulfate at the 15β position (48, 55).

1.3.5. *Cytochrome P450 2D subfamily*

Members of the cytochrome P450 2D subfamily have been isolated from rats (56), mice (57) and humans (58, 59). They catalyze the oxidation of debrisoquine and a large number of drugs such as bufurolol, sparteine, and propanolol (60). The metabolism of debrisoquine is polymorphic in humans because of the absence of the cytochrome P450 2D6 protein in 5 to 10% of the population (61). Studies have shown that there are at least five genes in this subfamily, designated as 2D1, 2D2, 2D3, 2D4, and 2D5, present in rats and they code for

proteins that possess 75 % to 95 % amino acid similarity (62). Recent studies suggested that there is a correlation between deficiencies in cytochrome P450 2D catalytic activity and risk for various types of cancer, including those of the lung (63, 64), bladder (65) and breast (66). More extensive future studies are required to establish the role of cytochrome P450 2D in carcinogenesis.

1.3.6. *Cytochrome P450 2E subfamily*

The rat (67), human (67), and rabbit (68) 2E1 genes have been isolated and sequenced, and their deduced amino acid sequences are about 80% similar (69). A single form of this subfamily has been identified in rats and humans (67, 70), whereas two highly homologous forms exist in rabbits (71). Cytochrome P450 2E metabolizes chemicals of small molecular size, such as diethyl ether, halothane and N,N'-dimethylnitrosamine (27). Cytochrome P450 2E1 expression can be induced by exposure of animals to compounds including ethanol, acetone or isoniazid (24). Changes in physiological status such as starvation and diabetes also elevate the enzyme level (24).

1.3.7. *Cytochrome P450 3A subfamily*

In rats, two isoforms have been identified, namely cytochrome P450 3A1 and cytochrome P450 3A2. The isozymes are 89% similar in their amino acid sequences (69). Cytochrome P450 3A1 and cytochrome P450 3A2 are immunochemically crossreactive. Cytochrome P450 3A proteins catalyze the metabolism of compounds such as ethylmorphine, erythromycin, mephenytoin and testosterone (27). Expression of the two isozymes appears to be regulated differently. Cytochrome P450 3A1 is undetectable in untreated male or female

rats (72). Cytochrome P450 3A2, on the other hand, is expressed constitutively in immature and mature male rats, and in immature female rats (72). Expression of both cytochrome P450 3A1 and cytochrome P450 3A2 can be induced by treatment with compounds such as pregnenolone-16α-carbonitrile (PCN), dexamethasone (DEX), and PB (72). In humans, the cytochrome P450 3A subfamily represents the most abundantly expressed set of cytochrome P450 isozymes in the liver. The human subfamily consists of four closely related proteins and accounts for about 60% of total cytochrome P450 (73). A member of this subfamily, cytochrome P450 3A7, is expressed in the fetal liver only, while another isoform, cytochrome P450 3A5, is found in adults but only in 25% of liver specimens tested (74). Cytochrome P450 3A4 is the most important isozyme in this subfamily. It is responsible for the metabolism of many clinically important drugs such as corticosteroids, antifungal agents, macrolide antibiotics, and antineoplastic agents (74).

1.3.8. *Cytochrome P450 4A subfamily*

Cytochrome P450 4A1 and cytochrome P450 4A3 were isolated in rats (75, 76). They share a 72% amino acid sequence similarity (76). A third gene, cytochrome P450 4A2, has been identified but its expression has not been verified (77). Cytochrome P450 4A isozymes metabolize lauric acid hydroxylation at the ω position (78) and are involved in the biotransformation of arachidonic acid (79). Peroxisomal proliferating agents such as clofibrate are inducers of cytochrome P450 4A isozymes (24).

Table 1.2. Inducers and substrates of hepatic cytochromes P450 in rats. Adapted from Goeptar *et al.*, 1995 (12).

Cytochrome P450	Inducers	Substrate
CYP1A subfamily		
CYP1A1	β-naphthoflavone, 3-MC, aroclor, TCDD	7-ethoxyresorufin, benzo[a]pyrene, benzo[a]anthracene, chrysene.
CYP1A2	isosafrole, 3-MC, aroclor β-naphthoflavone, TCDD	2-amino-3-methylimidazo[4,5-f]quinoline, 2-amino-3',5-dimethylimidazo[4,5-f]quinoline
CYP2B subfamily		
CYP2B1	phenobarbital, aroclor, DDT, dieldrin	7-pentoxyresorufin, olefins, acetylenes, 7,12-dimethylbenzo[a]anthracene testosterone (16β position)
CYP2B2	same as above	same as above
CYP2E subfamily		
CYP2E1	ethanol, ether, acetone, dimethylsulfoxide	N,N'-dimethylnitrosamine, ethanol, chlorzoxazone
CYP3A subfamily		
CYP3A1	pregnenolone 16α-carbonitrile, dexamethasone, phenobarbital	testosterone (6β position), ethylmorphine
CYP4A subfamily	clofibrate	prostaglandins

1.4. *INDUCTION OF CYTOCHROMES P450*

Induction is defined as *de novo* synthesis of an enzyme (24). The primary outcome of induction is an increase in the hepatic level of the enzyme with concomitant increase in the associated catalytic activity. Enzyme induction can be regulated at the level of transcription and/or posttranscription. Various types of cytochrome P450 inducers and their proposed mechanisms of induction will be discussed in this section.

1.4.1 *Cytochrome P450 1A Inducers*

Cytochrome P450 1A enzymes are induced primarily by compounds that are structurally related to 3-MC and TCDD (24). The most potent inducers of cytochrome P450 1A are coplanar polychlorinated biphenyls (PCBs) that possess two *para*, one or two *meta*, and no *ortho* chlorine substituents (80). Compounds such as phenothiazines, βNF, and plant indoles are also inducers of this subfamily (Table 1.2) (24).

Induction of cytochrome P450 1A1 is mediated by the aromatic hydrocarbon receptor (AhR) (81). AhR has been shown to exist in a wide variety of mammalian and nonmammalian tissues (81). In rodents, AhR is found in liver and extrahepatic tissues such as lung, kidney, spleen, and intestine (81). In humans, AhR has been detected in tissues including lymphocytes, placenta, and lung (81). Recent studies suggested that in addition to drug metabolism, AhR may also be involved in the regulation of cell growth and differentiation (81).

In the absence of a ligand, AhR is found in the cell cytoplasm. The ligand-binding subunit of AhR is complexed with the heat shock protein (hsp 90), and possibly with other proteins (81). In a typical induction process, TCDD or other AhR-receptor ligands enter the

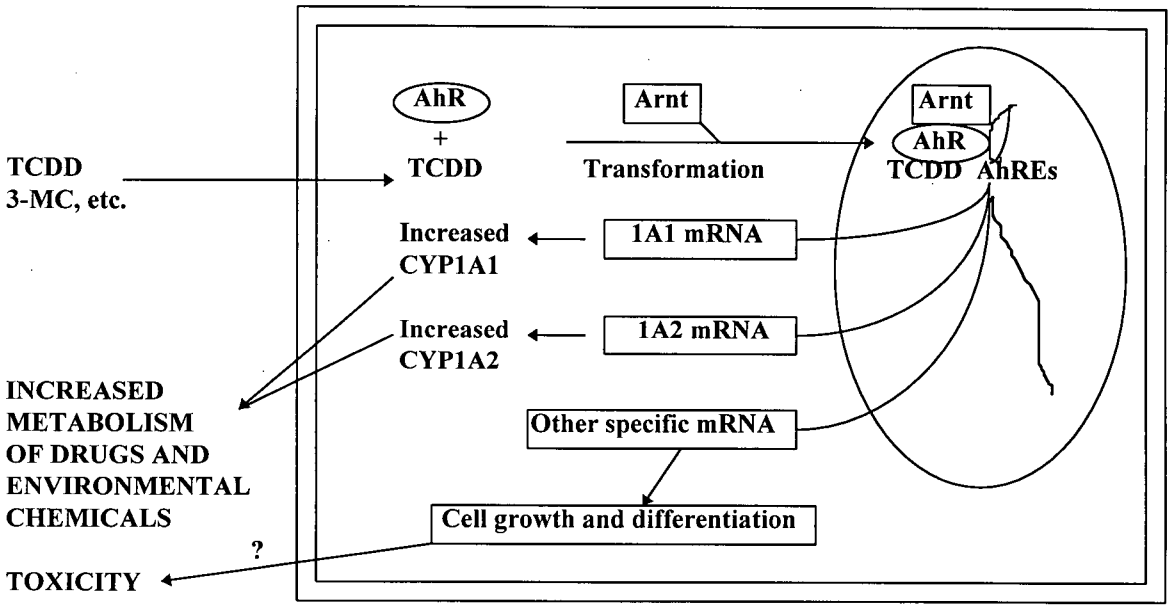


Figure 1.2. General model illustrating the Ah-receptor-mediated enzyme induction. Adapted from Okey *et al.*, 1990 (81).

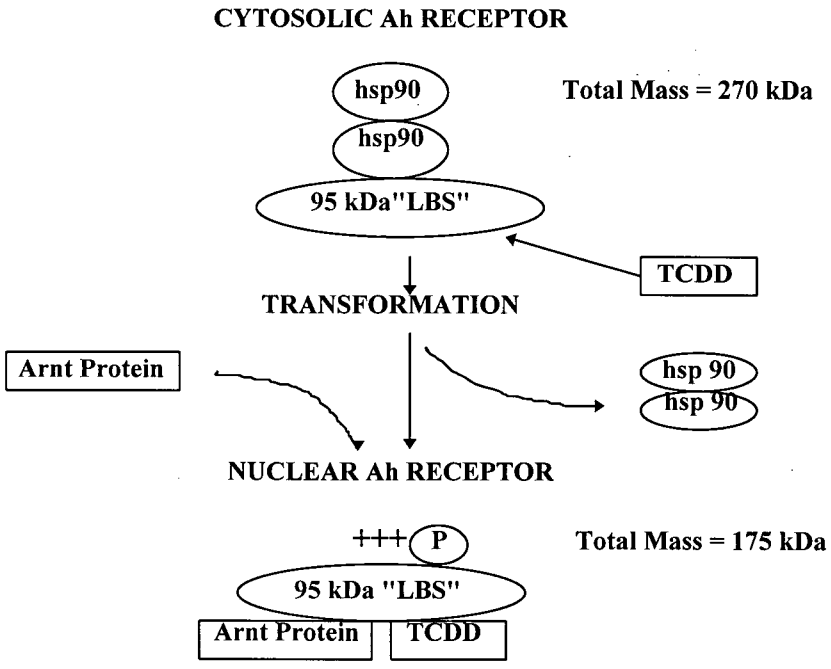


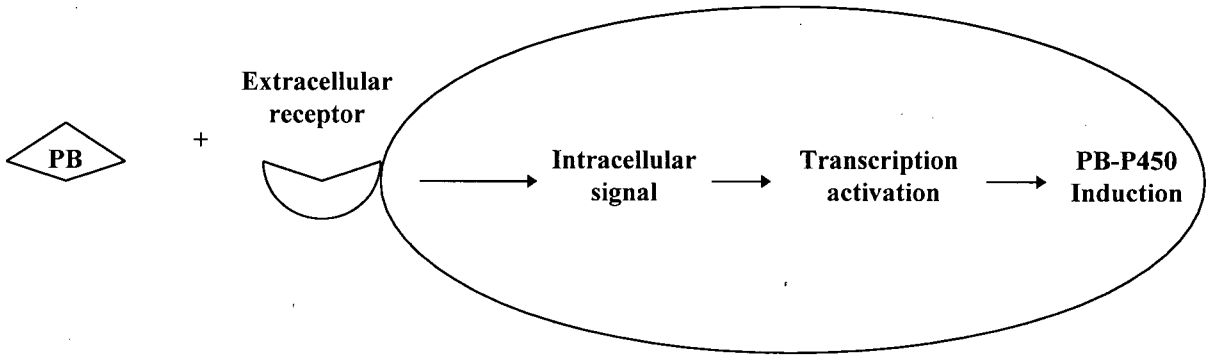
Figure 1.3. Postulated oligomeric structures of cytosolic and nuclear forms of the AhR and the sequence of events in transformation of cytosolic AhR to the DNA-binding state. The receptor may require phosphorylation in order to bind AhRE, which is indicated by "P". Adapted from Okey *et al.*, 1990 (81).

cell by diffusion and bind to the cytosolic AhR (Figure 1.2). The resulting inducer-receptor complex undergoes an energy-requiring transformation process, during which AhR releases hsp 90 and converts into a form that can associate with the 5' flanking region of DNA sequences, namely Ah-responsive elements (AhRE) (Figure 1.3) (81). The nuclear form of AhR is composed of a ligand binding subunit and Arnt protein (Ah receptor nuclear translocator protein). It is uncertain whether Arnt is a component of cytosolic AhR, or whether Arnt associates with AhR after hsp 90 is released during the transformation process. Binding of the nuclear AhR to AhRE, which may or may not require phosphorylation, results in transcriptional activation of cytochrome P450 1A1 and P450 1A2 genes (81), which in turn leads to an increase in cytoplasmic mRNA with a corresponding increase in microsomal cytochrome P450 1A1 and P450 1A2 proteins. In addition to cytochrome P450 enzymes, binding of the Ah receptor also induces phase II conjugating proteins such as uridine-diphosphate-glucuronosyltransferases, glutathione S-transferases and aldehyde dehydrogenases (81).

1.4.2. *Cytochrome P450 2B inducers*

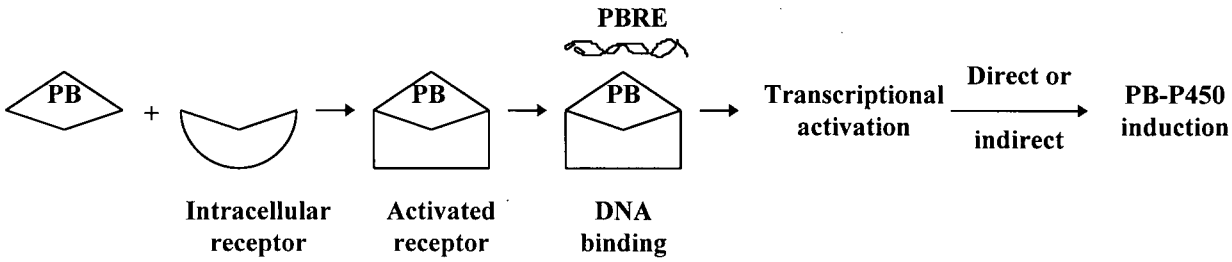
The major inducer of the cytochrome P450 2B subfamily is PB (Table 1.2). Halogenated hydrocarbons that do not possess coplanar aromatic structures, such as DDT, chlordanes, and some *ortho*-chlorine-substituted PCBs also induce cytochrome P450 2B (37). The induction process primarily involves transcriptional activation of cytochrome P450 2B genes, leading to accumulation of mRNA and increased cytochrome P450 2B enzymatic activities. At present, the mechanism by which the cell recognizes the inducer and conveys the information to the transcriptional machinery remains unknown. Thus far, several models and working hypotheses of the mechanism of cytochrome P450 2B induction have been proposed:

I. Receptor-dependent induction mechanisms. Adapted from Waxman *et al.* 1990 (37):



Scheme 1.

According to this model, PB binds to an extracellular receptor, leading to the formation of intracellular secondary messengers which in turn, activate transcription of cytochrome P450 genes (37)



Scheme 2.

Because of the lipophilic nature of PB and PB-like inducers, induction could be initiated by having PB diffuse through the plasma membrane and bind to an intracellular receptor (37). The resulting inducer-receptor complex could undergo a conformational change into a form that binds to a PB-responsive element in target genes (PBRE) leading to their transcriptional activation (37).

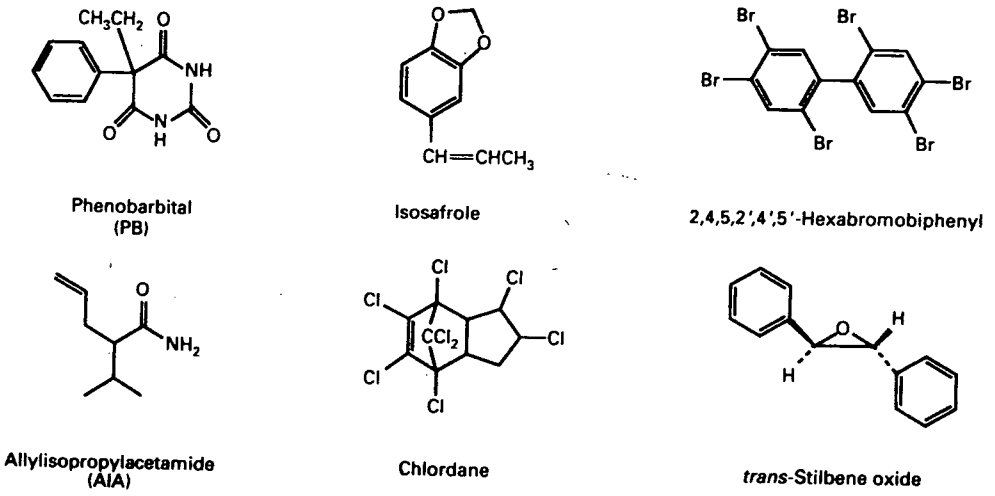


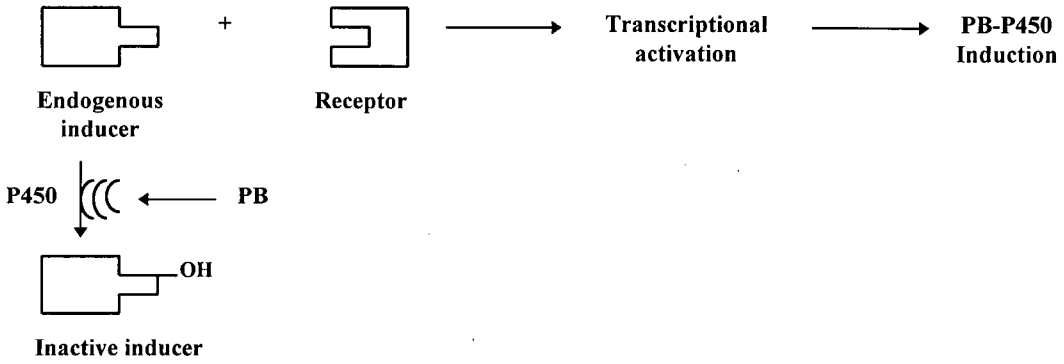
Figure 1.4. Structural diversity of phenobarbital-like inducers. Adapted from Waxman *et al*, 1992 (37).

An attempt has been made in recent studies to elucidate the mechanism of PB-induced transcriptional activation. In *Bacillus megaterium*, the upstream regulatory region of the barbiturate-inducible CYP102 (BM-3) gene contains a 17-base pair element (designated as the "Barbie Box") that appears to be involved in gene regulation by PB (82, 83, 84). The presence of consensus Barbie Boxes has been detected in rat cytochrome P450 2B genes and several bacterial genes (83, 84). Studies indicated that the consensus sequences bound proteins from both bacterial and rat liver nuclei in a PB-dependent manner *in vitro* (82, 83, 84). At present, the function of the regulatory element and its significance in cytochrome P450 induction are being investigated.

A receptor-dependent induction mechanism is common to activation of various endogenous compounds such as steroid hormones and retinoic acid, and exogenous compounds such as TCDD. However, because of the great structural diversity among the PB-type inducers (Figure 1.4) (24), it is difficult to reconcile with the existence of a specific "PB receptor". Thus

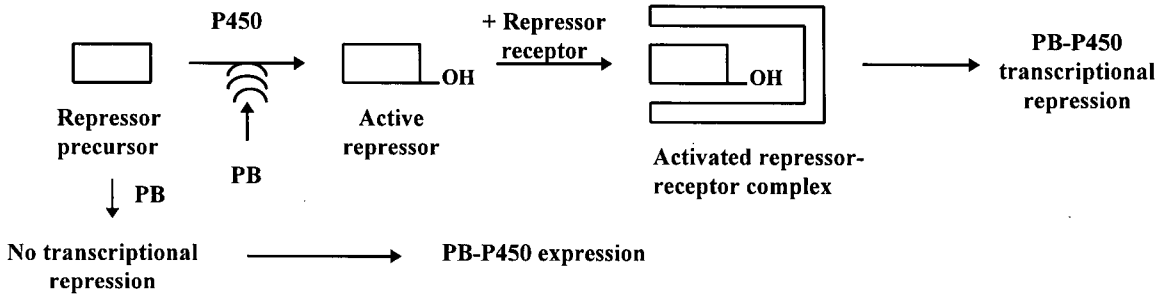
far, transcriptrional activation of cytochrome P450 2B genes by receptor-dependent mechanism has not been demonstrated because a receptor for PB has not been identified. Meanwhile, several alternative PB-receptor-independent mechanisms have been suggested.

II. Cytochrome P450-dependent induction mechanisms. Adapted from Waxman *et al.*, 1990 (37):



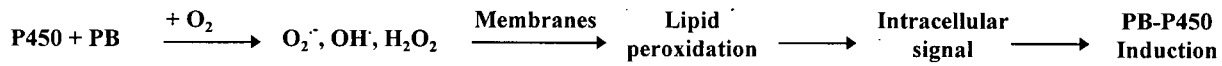
Scheme 3.

This model hypothesized that the induction process might be mediated by an endogenous substrate of cytochrome P450 2B, which is normally present at a very low level due to its metabolism by basal cytochrome P450 2B2 (37). Binding of PB to cytochrome P450 2B2 would block the metabolism of the endogenous substrate, causing an increase in its steady state concentration beyond the threshold required for transcriptional activation of cytochrome P450 2B genes (37).



Scheme 4.

According to this hypothesis, an endogenous steroid might serve as a "repressor precursor" that requires metabolism by basal cytochrome P450 2B enzymes in order to be functionally active (37). Binding of PB to cytochrome P450 2B enzymes would prevent formation of the active repressor (37), leading to derepression of cytochrome P450 2B transcription.



Scheme 5.

An alternative model suggested that the presence of PB might stimulate NADPH oxidation that is not coupled to substrate hydroxylation but linked to non-productive oxygen activation (37). This can lead to formation of reactive reduced oxygen species which in turn, can stimulate lipid peroxidation. The resulting peroxides or their secondary metabolites could be involved, directly or indirectly, in the induction process (37).

In addition to the models outlined above, other hypotheses such as stimulation of cytochrome P450 2B expression by alterations of membrane fluidity (85), or by changes of osmotic pressure (86), have been proposed. However, none of the models are supported by sufficient experimental evidence and the mechanism of cytochrome P450 2B induction still remains to be resolved.

1.4.3. *Cytochrome P450 2E inducers*

Expression of cytochrome P450 2E can be induced by ethanol (87) and related compounds such as acetone (88), isoniazid (89) and pyrazole (88). Alteration of an animal's physiological state such as fasting and diabetes has also been shown to induce expression of

cytochrome P450 2E1 (90, 91). The induction process appeared to be post-transcriptional, involving both protein stabilization and mRNA stabilization (24).

1.4.4. *Cytochrome P450 3A inducers*

Cytochrome P450 3A isozymes can be induced by a diverse group of compounds, including steroid antagonists, endogenous and synthetic glucocorticoids, macrolide antibiotics, various antifungal agents and "PB-like" compounds (Table 1.2) (24). Inducers of cytochrome P450 3A can be divided into two classes according to their inducing potency. The first class contains compounds that have a dramatic inductive effect on both cytochromes P450 3A1 and P450 3A2 (72); they include DEX, triacetyloleandomycin (TAO) and PCN (72). The second class contains compounds that preferentially induce one cytochrome P450 3A isozyme over another (72). For instance, both rifampicin and PB increase cytochrome P450 3A1 levels and have a lesser effect on cytochrome P450 3A2, whereas 3-MC induces cytochrome P450 3A2 but has no effect on cytochrome P450 3A1 (72). The mode of cytochrome P450 3A induction varies with different inducers. While DEX and PCN induce enzyme expression by transcriptional activation (92), mRNA and protein stabilization are involved in the induction produced by TAO and PB-like compounds (93). Other factors such as the age and sex of the animals are important determinants of the effects of various inducers on cytochrome P450 3A expression (72).

1.4.5. *Cytochrome P450 4A inducers*

Prototypical inducers of cytochrome P450 4A expression include clofibrate and related hypolipidemic drugs (78, 94), as well as phthalates used in plasticizers (94). An increase in liver weight and a proliferation of peroxisomes, followed by an elevation of peroxisomal enzymes are among the inductive effects of these compounds (24), hence the term, peroxisome proliferators. The mechanism of induction had been suggested to be receptor-mediated. A member of a nuclear hormone-receptor family that can be activated by peroxisome proliferators has been identified recently. The receptor, peroxisome proliferator activated receptor (PPAR), can also be activated by endogenous fatty acids (95). Upon activation, the receptor mediates the transcription of responsive genes through binding to DNA sequence peroxisome proliferator response elements (95). Studies have shown that purified PPAR requires an additional factor in order to bind to DNA, and one of the factors was shown to be the retinoid X receptor (95). The result of gene activation is an increase in the activity of cytochrome P450 4A isozymes, as well as an increase in peroxisomal β-oxidation of fatty acids (Figure 1.5) (95).

1.4.6. *Mixed inducers*

Studies have shown that certain compounds are able to induce more than one subfamily of cytochrome P450 forms. The term, "mixed inducer", indicates an agent that induces cytochrome P450 expression with a pattern similar to that obtained by simultanous administration of 3-MC and PB (24). Examples of mixed inducers are congeners of polybrominated and polychlorinated biphenyls. The inducing potency on the two cytochrome P450 subfamilies varies with the structure of the inducing compound. It was found, for

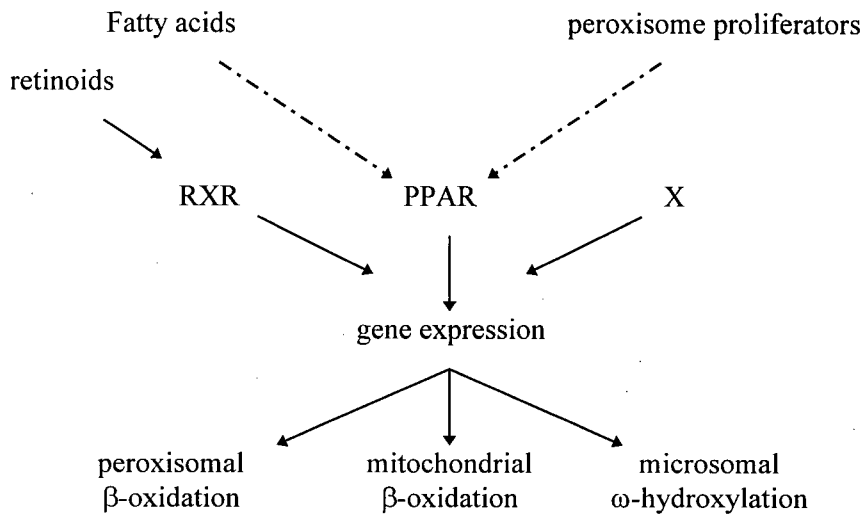


Figure 1.5. Fatty acids and peroxisome proliferators activate peroxisome proliferator activated receptors (PPAR), which together with retinoid X receptors (RXR) or other factor (X), can influence expression of genes involved in fatty acid degradation. Adapted from Gearing *et al.* 1994 (95).

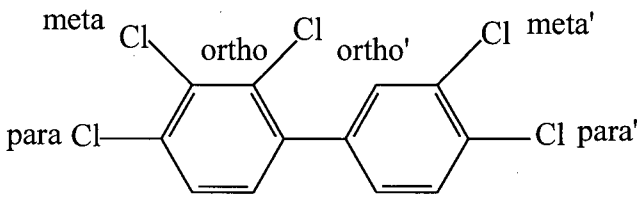


Figure 1.6. Chemical structure of a PCB with a mono-*ortho* substituent.

instance, that PCBs with mono-*ortho*-substituents had an inductive effect on cytochrome P450 1A and 2B isozymes (Figure 1.6) (24), whereas halogenations at additional *ortho* positions allows the compound to acquire PB-like inducing properties (24). The structure-activity relationship of these compounds is important in the study of the toxicological effects of environmental contaminants on hepatic cytochrome P450 expression, a topic that will be discussed in detail in the next section.

1.5. *USE OF CYTOCHROME P450 INDUCTION AS A BIOMARKER OF ENVIRONMENTAL CONTAMINATION*

Numerous studies have indicated that induction of cytochromes P450 seems to be a characteristic response in animals exposed to halogenated environmental contaminants. Halogenated environmental contaminants are anthropogenic organic compounds that include pesticides such as lindane, chlordane and dieldrin, industrial compounds and byproducts of various industrial processes such as hexachlorobenzene and PCBs (96). Some of these chemicals are extremely resistant to degradation by hydrolysis, free radicals, photolysis, and other environmental chemical reactions (97), and hence have long environmental half-lives. In addition, they are highly lipophilic and tend to accumulate in fatty tissues of organisms, leading to significant bioaccumulation (2). Many of these compounds act as mixed inducers and are able to induce both cytochrome P450 1A and cytochrome P450 2B isozymes (3). Induction of cytochrome P450 activities has proven to be a valid biomarker of exposure to potentially toxic xenobiotics in various species (98, 99, 100). In a recent study, cytochrome P450 induction was evaluated as a biomarker of exposure to environmental contaminants in the arctic ecosystem (2).

1.5.1. *Environmental contamination in the Arctic*

The release of chemicals into various ecosystems from industry, agriculture and forestry has a significant toxicological impact on the environment. A significant percentage of contaminants that are released into the environment at middle and lower latitudes migrate to northern regions via long-range atomospheric transport, waterways anad ocean currents (101). As a result, increasing amounts of various environmental contaminants have been found in circumpolar Arctic regions in the past 20 years. They have been detected in snow, ice, water, and air, and have contaminated every level of the arctic food chain.

The food chains of the arctic marine ecosystem include phytoplankton-zooplankton-fish-seal-polar bear, or phytoplankton-zooplankton-whale (101). Lipophilic contaminants are passed from prey to predator, eventually leading to their bioaccumulation and biomagnification in higher tropic levels of the food chain (Figure 1.7) (102, 103). For instance, mean PCB concentrations reported for pooled adipose tissue of polar bears from the Canadian arctic were 6- to 15-fold greater than those measured in blubber of ringed seals from the same geographic location (104). Furthermore, PCB levels greater than 70 μg/g of fat have been found in polar bears from both Canada and Norway (105, 106). In addition to marine and wildlife species, organochlorine pollutants have also been detected in humans (107). For cultural and economic reasons, some members of Inuit communities, and communities of other northern indigenous people, in the Canadian Arctic still consume large amounts of traditional foods that include ringed seal, walrus, beluga whale, narwhal meat and muktuk (beluga skin and blubber) (108). A study has shown that greater than 10% of the population in Broughton Island, N.W.T, had a diet that exceeded the tolerable daily intake of 1 μg/kg/day PCBs established by the

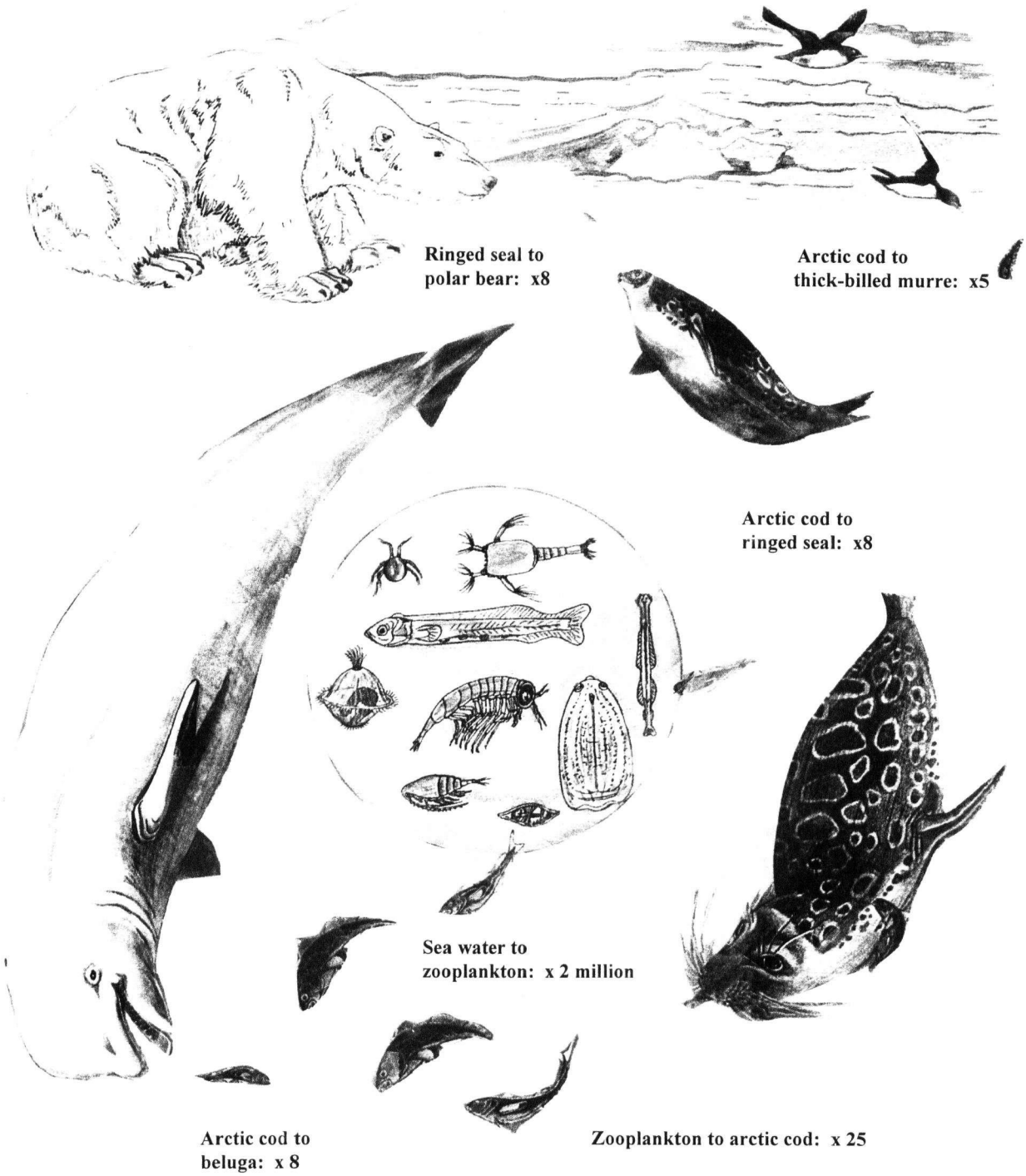


Figure 1.7. Biomagnification of PCBs in the Arctic. Adapted from Canadian Geographic, 1991 (102). Many chemicals become more concentrated and toxic as they move up the food chain. In the Arctic, PCBs in sea water accumulate in the fatty tissues of zooplankton, increasing in concentration by two million times. The biomagnification continues at each link in the food chain, resulting in a bigger dose for animals at the top. The level of PCBs in polar bears is about three billion times greater than in the Arctic Ocean (102).

government of Canada (108). Levels of PCBs and other contaminants in milk of Inuit women in northern Quebec have also found to be two- to ten-fold greater compared to women in southern regions (96, 109). Toxic effects of organochlorines have been demonstrated in both wildlife and humans. These chemicals have been shown to cause reproductive abnormalities, organ damage, hormonal alteration, immune suppression and tumors in beluga whales (110). In humans, organochlorines are linked to increased risk of low birth weight, and disorders of ectodermal tissues in offspring, including various endocrine, immunological and neurological abnormalites (107). Because of the significant impact of organochlorine xenobiotics on the environment, wildlife and humans, it is essential to routinely monitor and assess toxicological contamination.

1.5.2. *Enzyme induction in polar bears as a biomarker of envionment contamination*

The influence of several organochlorine contaminants on the induction of hepatic cytochromes P450 in polar bears and the potential use of immunoquantitation as a bioindicator of organochlorine contaminant exposure in the arctic marine ecosystem was explored in a recent study (2). Polar bears were considered to be an ideal sentinel species for several reasons. Polar bears are widely distributed throughout the arctic circumpolar regions with individual bears ranging over vast expanses of the frozen Arctic Ocean in search of food (111, 112). Polar bears are located at the top of the arctic food chain. They feed on ringed seals which in turn feed on Arctic cod (103). Their diet is high in fat, consisting mostly of seal skin and blubber, which serves as a depot for accumulation of lipophilic xenobiotics (103). Consequently, biomagnification of organochlorine compounds occurs in polar bears (103). As the Inuit also

eat marine mammals including seals and occasionally bears, their diet is high in fat, and hence the polar bear is well suited as a surrogate for human exposure.

Polar bear hepatic cytochromes P450 were characterized in a previous study according to their immunological relatedness to rat cytochromes P450 (2). Polar bear hepatic microsomes cross-reacted with antibodies raised against rat cytochromes P450 1A, P450 2B, P450 2C and P450 3A (2), indicating that immunorelated forms of cytochrome P450 exist in polar bear. Cytochrome P450 1A and P450 2B levels appeared to be elevated in polar bear liver (2), and the cytochrome P450 1A content was found to correlate strongly with organochlorine residue concentrations in liver (3). The results suggested that induction of polar bear cytochromes P450 had occurred as a result of exposure to environmental contaminants (3). However, a confounding factor in the study was that the bears had been immobilized with Telazol® 0.5 to 11 days prior to death. Therefore, it was essential to determine whether treatment with Telazol® had any effect on hepatic cytochrome P450 expression.

1.5.3. *Use of immobilizing agents in polar bears*

Since the late 1960's, many agents have been tested for immobilization of polar bears for population studies. The first agent tested was succinylcholine chloride (113). Succinylcholine was used in black and brown bears, however, it had produced little effect and caused a relatively large number of deaths in polar bears, and its use was quickly discontinued (113). Soon afterward, phencyclidine hydrochloride (Sernylan®) was tried. It appeared to immobilize the bears reasonably well (113). The main disadvantage of phencyclidine was its ability to produce convulsions and respiratory depression in polar bears (113). The sale of

phencyclidine was later banned because of its hallucinogenic properties in humans (114). A combination drug Rompun®, consisting of xylazine hydochloride and ketamine hydrochloride in a 1:1 ratio, produced optimal immobilization in polar bears (113); however, this drug could cause significant cardio-respiratory depression that prevented thermoregulaton in the bears (113). Other agents such as etorphine hydrochloride (M99®) and carfentanil (Carfentanil®) were also tested (115). All these drugs or drug combinations exhibited one or more problems in terms of safety to the bears or researchers.

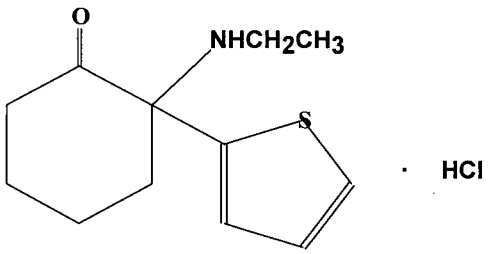
Because of promising results from preliminary studies of Telazol® as an immobilizing agent in various species (1), Telazol® was tested in polar bears. The results indicated that the drug was ideal for polar bears. The animals were immobilized rapidly, they showed a wide dose tolerance, they were able to thermoregulate, and they recovered quickly from the drug effect (113). Thus, Telazol® had shown to be the best immobilizing agent for polar bears, and is being used almost exclusively (113).

1.6. *TELAZOL®, TILETAMINE, AND ZOLAZEPAM*

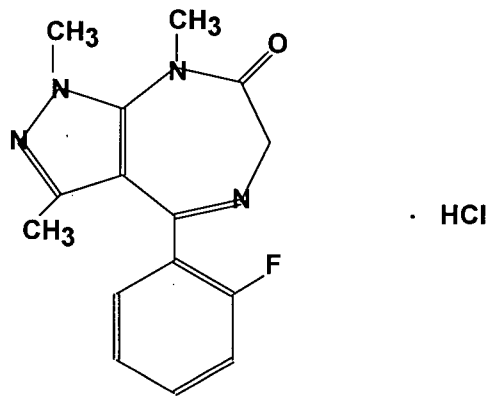
Telazol® (Fort Dodge, Iowa) is a 1:1 (w:w) combination of tiletamine hydrochloride (2-[ethylamino]-2-[2-thienyl]-cyclohexanone hydrochloride) and zolazepam hydrochloride (4-[*o*-fluorophenyl]-6,8-dihydro-1,3,8-trimethylpyrazolo[3,4-e][1,4]diazepin-7[1H]-1-hydrochloride) (Figure 1.8). Telazol® is officially approved for use as an injectable anesthetic in cats and dogs in the United States, while in Canada, it is listed as an experimental drug and has not been approved for routine use. However, Telazol® has been used extensively in a wide variety of wild and domestic animal species (1). The pharmacological advantages of Telazol®, which

include rapid induction of anesthesia, excellent muscle relaxation and smooth recovery (1), are ideal for veterinary diagnostic examinations, and various dental and minor surgical procedures. Telazol® has also proven to be an excellent immobilizing agent for field studies involving wild animals such as black bears (116), lions (117), jaguars (118) and polar bears (119). The drug is absorbed rapidly, produces an effective level of anesthesia, is relatively safe for the handlers and has a wide margin of safety (1). Adverse effects that have been associated with the use of Telazol® include excessive salivation, emesis and prolonged recovery (120). Factors such as the species, size, age, gender, temperament and physiological status of the animal are all important determinants of the pharmacological and adverse effects of Telazol®, and they should be taken into consideration when choosing an appropriate dose of the drug for different animal species. Table 1.3 compares the dose requirement of Telazol® with several commonly used anesthetic agents in various exotic animals.

Tiletamine is a constituent of Telazol®. It belongs to the class of "dissociative anesthetics" that includes ketamine and phencyclidine (122). Dissociative anesthetics appear to function by selectively interrupting association pathways to the brain before producing somatic sensory blockade (122). They are characterized by active cranial and spinal reflexes during anesthesia (122). The pharmacological activities of tiletamine include rapid induction, profound analgesia, normal pharyngeal-laryngeal reflexes, and cataleptoid anesthesia (122). At low doses, tiletamine antagonizes *N*-methyl-D-aspartate (NMDA) receptor-mediated excitation and produces anticonvulsant activity (123). At high doses, however, it is able to induce convulsions by interacting with non-NMDA receptors (123). Adverse effects of tiletamine include muscular clonus and body rigidity, hence, tiletamine is a poor muscle-relaxant when



Tiletamine HCl



Zolazepam HCl

Figure 1.8. Chemical structures of tiletamine hydrochloride and zolazepam hydrochloride.

Table 1.3. Dosage requirement of Telazol® and other anesthetic agents in various exotic animal species. Adapted from Carroll, 1989 (121).

Species	Ketamine	Fentanyl-dropericol (Innovar-Vet®)	Pentobarbital	Xylazine	Telazol®
Primates	5-10 mg/lb IM	0.04 mL/lb IM	9-13 mg/lb IV		
Exotic cats	5-20 mg/lb IM	0.05 mL/lb IM		0.5-1.0 mg/lb IM	2-15 mg/lb IM
Skunk	15 mg/lb IM	0.1 mg/lb IM	15-20 mg/lb IP		5-15 mg/lb IM
Raccoon	15 mg/lb IM	0.1 mL/lb IM	15-20 mg/lb IP		5-15 mg/lb IM
Ferret	15 mg/lb IM	0.1 mL/lb IM	15-20 mg/lb IP		
Rabbit	20 mg/lb IM	0.1 mL/lb IM	8-10 mg/lb IM		10-20 mg/lb IM
Guinea pig	20 mg/lb IM	0.06 mL/lb IM	0.03-0.05 mg/g IP		20-30 mg/lb IM
Rat	20 mg/lb IM	0.09 mL/lb IM	0.03-0.05 mg/g IP		10-20 mg/lb IM
Mouse	20 mg/lb IM	0.001 mL/lb IM	0.03-0.05 mg/g IP		
Gerbil, hamster	20 mg/lb IM		0.03-0.05 mg/g IP		20-30 mg/lb IM
Squirrel					2-10 mg/lb IM
Birds:					
Parakeet	0.1-0.2 mg/g IM		0.3-0.5 mg/g IM	50-75 mg/lb IM	
Parrot	0.1-0.2 mg/g IM		produces anesthesi	produces sleep of	10-20 mL/lb IM
Pigeon	0.02-0.1 mg/g IM		of 30 min duration	15-30 min duration	15-30 mg/lb IM
Chicken	0.02-0.05 mg/g IM				
Duck	0.02-0.05 mg/g IM				
Snake	25-50 mg/lb IM		10-15 mg/lb IP, IM		5-20 mg/lb IM
Lizard	25-50 mg/lb IM		10-15 mg/lb IP, IM		5-15 mg/lb IM
Turtle	25-50 mg/lb IM		6-10 mg/lb, IP, IM		2-10 mg/lb IM

used alone (122). Plasma half-lives of tiletamine in cats, dogs, monkeys, and rats are 2-4 h, 1.2 h, 1-1.5 h, and 30-40 min, respectively (122). Three metabolites, 2-(ethylamino)-2-(2-thienyl) cyclohexanol, 2-amino-2-(2-thienyl) cyclohexanol, and 2-amino-2-(2-thienyl) cyclohexanone have been isolated from cat urine (122).

Zolazepam is another constituent of Telazol®. It is a benzodiazepine derivative pharmacologically similar to chlordiazepoxide and diazepam (122). Zolazepam is an excellent anticonvulsant, anxiolytic and muscle relaxant (124). Zolazepam was investigated initially as a potential drug for humans and is now the only benzodiazepine approved officially for use in animals, but only in combination with tiletamine (122). Plasma half-lives of zolazepam in cats, dogs, rats, and monkeys are 4.5 h, 4-5 h, 3 h and 1 h, respectively (122). The metabolites found in dogs included 1-dimethyl-zolazepam and its hydroxylated derivative (125). In rats, 8-demethyl-6-hydroxy-zolazepam was the main metabolite isolated, while the 1-demethyl-zolazepam metabolite was found in female rats only (125), indicating a sex-difference in the metabolism of this drug. When zolazepam is used in combination with tiletamine, it prevents tiletamine-induced convulsion, produces optimal muscle relaxation, and provides smooth emergence from anesthesia (122). The use of tiletamine and zolazepam in a 1:1 (w:w) ratio (Telazol®) is ideal in enhancing the pharmacological activities of both agents while minimizing the adverse effects associated with the use of either agent alone (122).

1.7. *STRUCTURE ANALOGS OF TILETAMINE AND ZOLAZEPAM AND THEIR EFFECTS ON HEPATIC CYTOCHROMES P450*

Very little is known about the effect of Telazol®, tiletamine or zolazepam, or related drugs on the cytochrome P450 system. An earlier study indicated that ketamine, a dissociative anesthetic structurally related to tiletamine, produced an inductive effect similar to that of PB. Both treatments significantly increased total cytochrome P450 content, benzphetamine *N*-demethylase, benzo[a]pyrene hydroxylase and cytochrome c reductase activities, with ketamine being the less potent of the two drugs (126). Treatment with benzodiazepines including oxazepam and chlordiazepoxide had been shown to produce a dose-dependent increase in liver weight, hepatic protein content and total cytochrome P450 content in rodents (127, 128, 129, 130). In a study involving mice, high doses of dietary oxazepam elicited an increase in EROD, BROD, and PROD activities of 6-fold, 50-fold, and 28-fold, respectively (131). Oxazepam appeared to produce a pattern of induction of cytochromes P450 similar to that produced by PB (131, 132). On the other hand, variable results were obtained for the effect of diazepam on hepatic microsomal monooxygenase enzymes. While some studies suggested that diazepam elevated liver weight in rats (127) and induced cytochrome P450 content and aminopyrine *N*-demethylase activities in mice (129), other studies showed that it had little or no inductive effect on hepatic enzyme activities (133). Thus, it appeared that benzodiazepines differ from each other in regard to their effect on cytochrome P450 expression, and high doses coupled with repeated treatments are required to elicit induction of cytochrome P450 enzymes. Currently, there is no information in the literature regarding the effect of tiletamine or zolazepam on cytochrome P450 expression. Hence, the present study will examine the effects of treatment with Telazol®, tiletamine, and zolazepam on hepatic cytochromes P450 in rats.

1.8. *HYPOTHESIS OF THE PRESENT STUDY*

The hypothesis of the present study was:

Telazol®, at a dose greater than that used routinely in a clinical setting or in field studies, would have an inductive effect on hepatic cytochrome P450 expression.

This hypothesis is based on the previous observation that analogs of tiletamine and zolazepam, namely ketamine and benzodiazepine derivatives such as diazepam and oxazepam, at doses greater than those used routinely, showed some evidence of cytochrome P450 induction. In addition, it is possible that use of a dissociative anesthetic in combination with a benzodiazepine might elicit a synergistic effect on hepatic enzyme expression.

1.9. *OBJECTIVES OF THE PRESENT STUDY*

The overall goal of the present study was to determine the effects of Telazol®, tiletamine, and zolazepam on expression of hepatic cytochrome P450 isozymes, using rat as the experimental model. Specific objectives of the project were:

1. To investigate the effects of acute treatment (single injection) with increasing doses of Telazol® on hepatic cytochrome P450-mediated enzyme activities and protein levels.
2. On the basis of positive results obtained in Objective 1, to determine the constituent(s) of Telazol® responsible for the observed hepatic cytochrome P450 induction.
3. To compare the induction pattern of cytochromes P450 expressed by Telazol®, tiletamine and zolazepam with that produced by PB using a similar dosing regimen.

The above objectives were accomplished by measuring the hepatic cytochrome P450 content, various cytochrome P450-catalyzed activities including *p*-nitrophenol hydroxylase, ethoxyresorufin, benzyloxyresorufin and pentoxyresorufin *O*-dealkylase, and testosterone hydroxylase activities, and hepatic protein levels of specific cytochrome P450 isozymes.

2. EXPERIMENTAL

2.1. *CHEMICALS*

Chemicals and reagents used in this study were obtained from the following sources:

Aldrich Chemical Company Inc. (Milwaukee, Wisconsin, U.S.A.):

Resorufin.

Anachemia (Mississauga, Ontario, Canada):

Hydrochloric acid.

BDH Chemicals (Toronto, Ontario, Canada):

Ethanol (95%); ethylenediaminetetraacetic acid, disodium salt (EDTA); Folin & Ciocalteu phenol reagent; magnesium chloride; methanol (ACS-grade); potassium chloride; potassium ferricyanide, sodium carbonate anhydrous; sodium hydroxide and sucrose.

Bio-Rad Laboratories. (Mississauga, Ontario, Canada):

Acrylamide 99.9%; N,N'-methylene-bis-acrylamide (BIS); 2-mercaptoethanol and N,N,N',N'-tetramethylethylenediamine (TEMED).

Boehringer Mannheim Canada Ltd. (Laval, Quebec, Canada):

Nicotinamide adenine dinucleotide phosphate tetrasodium salt (NADPH).

Carnation, Inc. (Toronto, Ontario, Canada):

Skim milk powder.

Fisher Scientific Ltd. (Vancouver, British Columbia, Canada):

HPLC-grade acetonitrile; ascorbic acid; HPLC-grade dichloromethane; glycerin; glycine; HPLC-grade methanol; perchloric acid; potassium phosphate monobasic; sodium chloride; sodium dodecyl sulphate (SDS) and sodium phosphate.

Fort Dodge Laboratories, Inc. (Fort Dodge, Iowa, U.S.A.):

Telazol®.

ICN Biomedicals Canada Ltd. (St-Laurent, Quebec, Canada):

Bovine serum albumin (globulin and fatty acid free, fraction V) and Tris(hydroxymethyl)aminomethane (Trizma base).

J. T. Baker Chemical Co. (Phillipsburg, New Jersey, U.S.A.):

Sodium dithionite.

Mandel Scientific Company Ltd. (Edmonton, Alberta, Canada):

Blotting paper and nitrocellulose membrane (Schleicher & Schwell).

Medigas Pacific (Vancouver, British Columbia, Canada):

Carbon monoxide gas (99.5% purity).

Molecular Probes, Inc. (Eugene, Oregon, U.S.A.):

7-benzyloxyresorufin; 7-ethoxyresorufin and 7-pentoxyresorufin.

Pierce (Rockford, Illinois, U.S.A.):

4-Nitro-blue tetrazolium chloride (NBT).

Schwarz/Mann Biotech. (Cleveland, Ohio, U.S.A.):

Ammonium persulfate (electrophoresis grade).

Sigma Chemical Co. (St. Louis, Missouri, U.S.A.):

Bromphenol blue; cupric sulfate pentahydrate; dimethyl sulfoxide; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); 1,2-dihydroxy-4-nitrobenzene (4-nitrocatechol); 4-nitrophenol (spectrophotometric grade); pyronin Y (certified); sodium potassium tartrate tetrahydrate; Tris(hydroxymethyl)aminomethane hydrochloride (Trizma HCl) and polyoxyethylene sorbitan monolaurate (Tween 20).

Steraloids Inc. (Wilton, New Hampshire, U.S.A.):

Testosterone; androstenedione and 2α-, 6β-, 7α-, 11β-, 16α- and 16β-hydroxytestosterone.

Immunocorp (Montreal, Quebec, Canada):

Affinity-isolated, alkaline-phosphatase conjugated, goat $F(ab')_2$ anti-rabbit IgG (gamma and light chain specific, human IgG adsorbed) from TAGO Immunologicals.

Xymotech Biosystems (Mt. Royal, Quebec, Canada):

5-bromo-4-chloro-3-indolyl phosphate (BCIP) disodium salt.

Dr. S. M. Bandiera (Faculty of Pharmaceutical Sciences, University of British Columbia, Vancouver, British Columbia, Canada):

Purified rat cytochrome P450 2B1; partially purified cytochrome P450 3A1; purified rabbit control IgG; polyclonal rabbit anti-rat cytochrome P450 1A1 IgG, polyclonal rabbit anti-rat cytochrome P450 2B1 IgG (backabsorbed and non-backabsorbed); polyclonal rabbit anti-rat cytochrome P450 3A1 IgG (backabsorbed); pooled liver microsomes prepared from ethanol-, isoniazid-, or phenobarbital-treated, adult male Long Evans rats.

Dr. H. A. Semple (College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan, Canada):

Tiletamine and zolazepam.

Dr. Paul E. Thomas (Department of Chemical Biology and Pharmacognosy, The State University of New Jersey, Rutgers, Piscataway, New Jersey, U.S.A.):

Polyclonal rabbit anti-rat cytochrome P450 2E1 IgG and monoclonal mouse anti-rat cytochrome P450 1A1 IgG.

2.2. *ANIMALS AND TREATMENT*

Animal treatment for this study was divided into three sets:

Set 1.

Adult (246-289 g) male Long Evans rats (Charles River Laboratories, Montreal, Quebec) were housed in polycarbonate cages on corncob bedding and maintained on 12 hours light and 12 hours dark cycles. The rats were allowed free access to food (PMI Chow, Richmond, Virginia) and water. After one week of acclimatization, rats received a single i.p. injection of Telazol® in distilled water at a dose of 20, 40, 80, or 120 mg/kg, while control rats received the vehicle at a dose of 1 mL/kg. Rats were decapitated 24 hours after treatment.

Set 2.

Adult (253-306 g) male Long Evans rats (Charles River Laboratories, Montreal, Quebec) were maintained under the same conditions as described above. Rats received a single i.p. injection of Telazol® at a dose of 120 mg/kg, tiletamine at a dose of 60 mg/kg, zolazepam at a dose of 60 mg/kg, or vehicle at a dose of 1 mL/kg. Animals were decapitated 24 hours after treatment.

Set 3.

Adult (253-313 g) male Long Evans rats (Charles River Laboratories, Montreal, Quebec) were maintained under the same conditions as described above. Rats received a single i.p. injection of phenobarbital at a dose of 60 mg/kg, 120 mg/kg, or vehicle at a dose of 1 mL/kg. Animals were decapitated 24 hours after treatment.

2.3. *PREPARATION OF HEPATIC MICROSOMAL FRACTION*

Hepatic microsomes were prepared from individual rats as described by Thomas *et al.* (134). Following decapitation, liver was removed quickly, weighed, minced, and placed in 20 mL of ice-cold 0.05 M Tris-HCl, 1.15 % KCl, pH 7.5, and homogenized with a Potter-Elvehjem glass mortar and a motor-driven pestle by 5 slow-speed passes with a loose-fitting pestle and 5 high-speed passes with a tight-fitting pestle. The homogenates were centrifuged at 9,000 x g for 20 min at 5°C in a Beckman centrifuge and the supernatant was filtered. The 9,000 x g supernatant was centrifuged at 105,000 x g for 60 min at 5°C. The glycogen was discarded and the resulting microsomal pellets were resuspended in ice-cold 10 mM EDTA, 1.15 % KCl, pH 7.4, using the homogenizer with 5 passes of the loose-fitting pestle, and centrifuged at 105,000 x g for 60 min at 5°C. The final pellets were resuspended in a small volume of (approximately 3 to 6 pellet volumes) of ice-cold 0.25 M sucrose by gentle homogenization. The microsomal preparations were aliquoted into a series of vials and stored at -80°C.

2.4. *DETERMINATION OF TOTAL CYTOCHROME P450 CONTENT*

Total cytochrome P450 content was determined from the carbon monoxide reduced difference spectrum using the method of Omura and Sato (4). Hepatic microsomes diluted in 0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM EDTA, were reduced with sodium dithionite and then saturated with carbon monoxide. Spectral measurements were performed using a SLM-Aminco DW-2 spectrophotometer. Total hepatic

cytochrome P450 content was calculated using a molar extinction coefficient of 91 $cm^2/mmol$ (135).

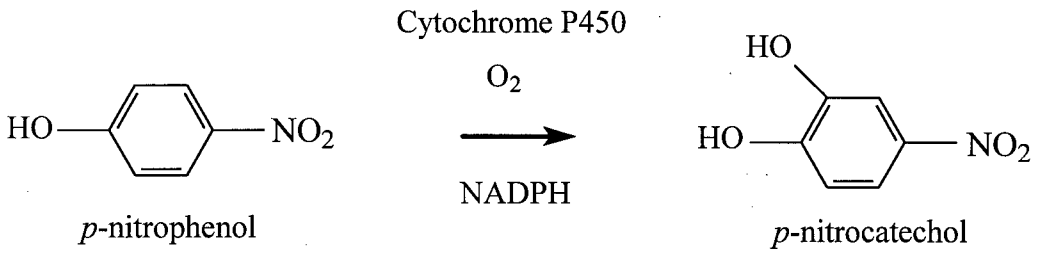
2.5. *DETERMINATION OF TOTAL PROTEIN CONCENTRATION*

Total hepatic protein concentration was determined by the method of Lowry *et al.* (136) using bovine serum albumin (BSA) as the standard. All samples were measured in duplicate at an absorbance of 650 nm.

2.6. *DETERMINATION OF CYTOCHROME P450-MEDIATED ENZYME ACTIVITIES*

2.6.1. *p-Nitrophenol hydroxylase assay*

The aromatic hydroxylation of *p*-nitrophenol results in the formation of *p*-nitrocatechol (1,2-dihydroxy-4-nitrobenzene), which is estimated colorimetrically. The reaction proceeds as shown below:



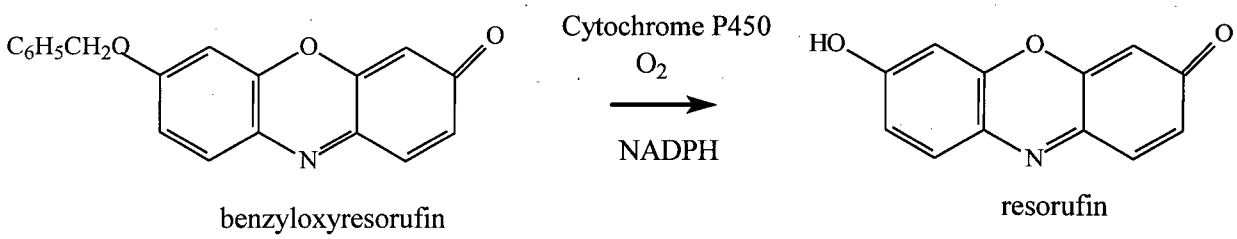
Scheme 1. Illustration of *p*-nitrophenol hydroxylation.

Hepatic microsomal *p*-nitrophenol hydroxylase activity was measured as described by Koop (137) and Reinke *et al.* (138). Reaction mixtures contained 1 mL of 0.2 M potassium phosphate buffer, pH 6.8, with 10 mM $MgCl_2$, 100 µL of 2mM *p*-nitrophenol, 100 µL of 20 mM ascorbic acid, and 0.4 mL of liver microsomal protein at a concentration of 10 mg/mL in a

final volume of 2 mL. After a preincubation period of 10 min at room temperature, reactions were initiated by the addition of 10 μL of 100 mM NADPH. The reactions were allowed to proceed for 5 min at 37°C and were terminated with 1 mL of 0.6 M perchloric acid. Tubes were mixed immediately and then spun for 10 min at 4000 rpm. The resulting supernatant was mixed with 0.2 mL of 10 M NaOH and incubated at room temperature for 15 min, after which the absorbance of the supernatant was measured at 543 nm using a Shimadzu UV-160 spectrophotometer. The amount of *p*-nitrocatechol formed was determined from a calibration curve.

2.6.2. *Benzyloxyresorufin O-dealkylase assay (BROD)*

The cytochrome P450 catalyzed dealkylation of benzyloxyresorufin is illustrated below:



benzyloxyresorufin resorufin

Scheme 2. Illustration of benzyloxyresorufin *O*-dealkylation.

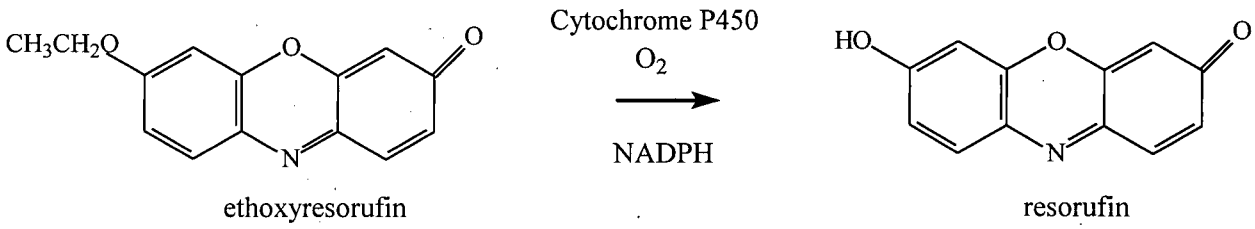
The BROD activity was measured according to the fluorimetric method of Burke *et al.* (41). Reaction mixtures contained 50 μL of hepatic microsomal protein at a concentration of 2 mg/mL, 1.93 mL of 0.1 M HEPES buffer, pH 7.8, with 5 mM $MgCl_2$, and 10 μL of 1 mM benzyloxyresorufin in a final volume of 1 mL. Reactions were initiated by the addition of 10 μL of 50 mM NADPH after a preincubation period of 2 min (experimental section 1) or 5 min (experimental sections 2 and 3) at 37°C. The fluorescence intensity was recorded before the

addition of NADPH and 2 or 5 min after the reaction was initiated. All measurements were made using a Shimadzu RF-540 spectrofluorophotometer interfaced with a Shimadzu DR-3 data recorder. The excitation and emission wavelengths were set at 530 nm and 584 nm, respectively, with slit widths of 2 nm. The amount of resorufin formed was determined from a standard curve of fluorescence intensity versus resorufin concentration.

In order to optimize operating conditions for BROD and PROD (described below) assays, preliminary studies were conducted as indicated above with varying microsomal concentrations and reaction time. Samples used for condition optimization were pooled liver microsomes prepared from untreated and phenobarbital-treated rats.

2.6.3. *Ethoxyresorufin O-deethylase assay (EROD)*

The cytochrome P450 catalyzed deethylation of ethoxyresorufin proceeds as follows:



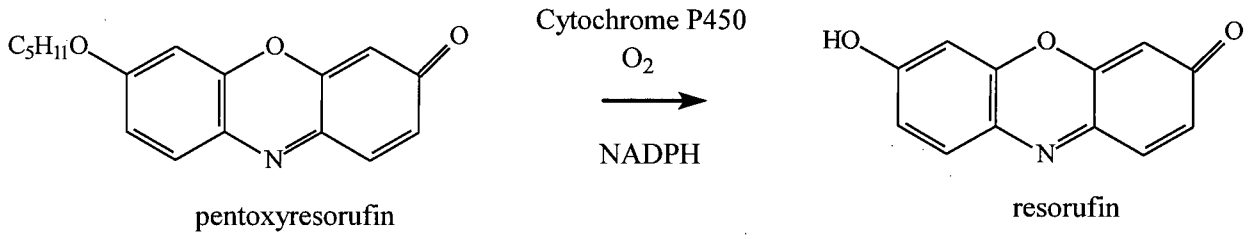
ethoxyresorufin resorufin

Scheme 3. Illustration of ethoxyresorufin *O*-deethylation.

The EROD activity was determined as described by Burke *et al.* (41). The method was essentially the same as indicated for BROD above except ethoxyresorufin was used as the substrate and a 5-min reaction period was allowed for all samples.

2.6.4. *Pentoxyresorufin O-dependentylase assay (PROD)*

The cytochrome P450 catalyzed depentylation of pentoxyresorufin is shown below:



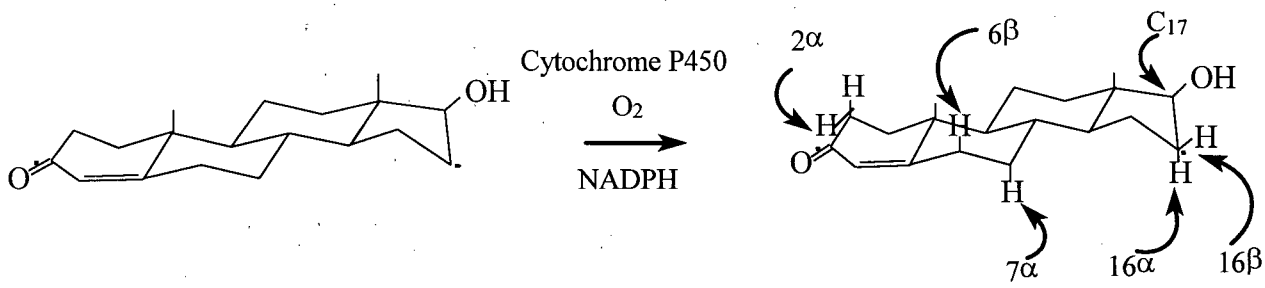
pentoxyresorufin resorufin

Scheme 4. Illustration of pentoxyresorufin *O*-dependentylation.

The PROD assay was performed as described by Lubet *et al.* (139), which was essentially the same as indicated for BROD above except pentoxyresorufin was used as the substrate.

2.6.5. *Testosterone hydroxylase assay*

The cytochrome P450-mediated hydroxylation of testosterone at various positions proceeds as follows:



Scheme 5. Illustration of testosterone 17-oxidation and 2α-, 6β-, 7α-, 16α- and 16β-hydroxylation.

Microsomal testosterone hydroxylase activities were determined by the method of Sonderfan *et al.* (53) with slight modifications. Reaction mixtures contained 50 μL of hepatic microsomal protein at a concentration of 6 mg/mL, 920 μL of 50 mM potassium phosphate buffer, pH 7.4, with 3 mM $MgCl_2$, and 10 μL of 100 mM NADPH in a final volume of 1 mL. Reactions were initiated by the addition of 20 μL of 12.5 mM testosterone in methanol after preincubating at room temperature for 10 min. The reactions were allowed to proceed for 5-min at 37°C and were terminated with 6 mL of methylene chloride. Each sample was spiked with 2.5 nmol of 11β-hydroxytestosterone in methanol and was mixed vigorously for 1 min. The aqueous and the organic phase were separated by centrifugation at 2000 rpm for 1 min. The aqueous layer was aspirated and discarded, while the organic layer was evaporated under a steam of nitrogen. The dried residue was reconstituted with 200 μL of HPLC-grade methanol and mixed for 1 min. The solution was subsequently filtered through a 13 mm, 0.45 μm, syringe filter and a 10 μL aliquot was analyzed by HPLC. Testosterone and its metabolites were resolved by at 40°C on a reverse phase C_{18} column using a Shimadzu LC-6A binary gradient HPLC system equipped with an SIL-6B autosampler, SPD-6A variable wavelength UV detector (set at 254 nm) and CTO-6A column heater (Shimadzu Scientific Instruments). A Supelcosil LC-18 octyldecylsilane column (5 μm particle size, 15 cm x 4.6 mm) was used and preceded by a Supelcosil LC-18 guard column (40 μm particle size, 2 cm x 4.6 mm) (Supelco, Bellefonte, PA). The column was eluted with a concave gradient from 90 % solvent A (methanol:water:acetonitrile, 39:60:1) to 85 % solvent B (methanol:water:acetonitrile, 80:18:2) over a period of 40 min at a constant flow rate of 1.5 mL/min (experimental section 1), or it was eluted using a solvent program which began with 100 % solvent A

(methanol:water:acetonitrile, 35:64:1:) from 0 to 10 min, followed by a liner gradient of solvent B (methanol:water:acetonitrile, 80:18:2) from 0 to 100 % from 10 to 29 min, to 100 % solvent B from 29 to 31 min, and a linear gradient to 100 % solvent A from 31 to 32 min followed by re-equilibration with 100 % solvent A from 32 to 34 min at a total flow rate of 2 mL/min (experimental sections 2 and 3). Testosterone and its metabolites, which include 2α-, 6β-, 7α-, 16α-, 16β- hydroxytestosterones and androstenedione, were identified by comparing retention times to those of authentic standards. The quantity of each metabolite formed was calculated from the slope of a linear calibration curve using the ratio of the peak area (integrated by a Shimadzu CR501 chromatography data processor) of the metabolite to that of the internal standard. Calibration curves were generated for each metabolite by plotting the ratio of the peak area of the authentic standard to that of the internal standard versus the concentration of the authentic standard. Calibration curves were prepared as part of every assay and employed four concentrations of each authentic standard. Tubes to which authentic standards were added contained the complete reaction mixture except for microsomal protein.

2.7. *ANTIBODY INHIBITION STUDIES*

BROD and PROD assays were performed as described above except that hepatic microsomes were incubated with increasing concentrations of rabbit anti-rat cytochrome P450 2B1 polyclonal IgG (non-backabsorbed) or control rabbit IgG for 5 min prior to initiation of the reaction by the addition of NADPH.

2.8. *PREPARATION OF ANTIBODIES*

Polyclonal antibody against rat cytochromes P450 1A1, P450 2B1, or P450 3A1 was raised in female New Zealand rabbits immunized with the electrophoretically homogenous protein. IgG was purified from a pool of heat-inactivated high-titer antisera derived from multiple bleedings from several rabbits using a combination of caprylic acid precipitation followed by ammonium sulfate precipitation and final cleanup on a DEAE-Sephacel column. IgG concentration was determined spectrophotometrically at 280 nm, E_{1cm} = 13 for a 1% solution, in phosphate-buffered saline, pH 7.4. The specificity of the antibody was assessed using Ouchterlony double diffusion analysis, noncompetitive enzyme-linked immunosorbent assay, and immunoblots with purified rat cytochrome P450 isozymes and with different rat liver microsomal preparations. Backabsorbed anti-cytochrome P450 2B1 IgG reacted with cytochrome P450 2B1, P450 2B2 and a third, noninducible member of the cytochrome P450 2B subfamily, but not with other cytochrome P450 isozymes. Anti-cytochrome P450 3A1 IgG reacted with both cytochromes P450 3A1 and P450 3A2 and several other cytochrome P450 proteins.

2.9. *SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (140) using a Hoefer SE 600 vertical slab gel unit. The discontinuous SDS-polyacrylamide gel consisted of a 3.0% acrylamide stacking gel (0.75 mm thick, 1 cm long) and a 7.5% acrylamide separating gel (0.75 mm thick, 12.5 cm long). The stacking gel contained 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 3% (w/v) acrylamide-BIS, 0.08% (w/v) ammonium persulphate and 0.05% (w/v) TEMED. The

separating gel contained 0.375 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 7.5% (w/v) acrylamide-BIS, 0.042% (w/v) ammonium persulphate and 0.03% (w/v) TEMED. Microsomes diluted to 0.5 mg/ml, in sample dilution buffer containing 0.062 M Tris-HCl (pH 6.8), 1% (w/v) SDS, 10% glycerol, 0.001% (w/v) bromophenol blue and 5% (v/v) mercaptoethanol, were boiled for 2 min. The denatured microsomal proteins (20 μl/well) were subjected to electrophoresis at a constant current of 0.12 mA per gel through the stacking gel and 0.24 mA per gel through the separating gel.

2.10. *IMMUNOBLOTS*

Proteins resolved by SDS-PAGE were transferred electrophoretically to nitrocellulose membranes according to the method of Towbin *et al.* (141), using a Hoefer Transphor Apparatus (Model TE 52) at a setting of 0.4 A for 2 h at 4 °C. The nitrocellulose membranes were placed in blocking buffer (1% BSA, 3% skim milk powder, in modified PBS) overnight at 4°C. The next day the blocking buffer was discarded and the membranes were washed 3 times with wash buffer (0.05% Tween 20 in modified PBS) at 10 min intervals. The membranes were incubated in antibody dilution buffer (1% BSA, 3% skim milk powder, 0.05% Tween 20, in modified PBS) containing primary antibody against rat cytochrome P450 at 37°C for 2 h with shaking. After washing with wash buffer 3 times at 10 min intervals, the membranes were incubated with antibody dilution buffer containing alkaline phosphatase-conjugated goat anti-rabbit secondary antibody at 37°C for 2 h with shaking. The nitrocellulose membranes were washed 3 times. Substrate solution (0.01% NBT, 0.005% BCIP, in 0.1 M Tris-HCl, 0.5 mM

$MgCl_2$, pH 9.5) was added to the membranes under subdued light. The reaction was stopped after a specific time period by immersing the membranes in distilled water.

For immunodetection of cytochrome P450 1A1, the concentration of primary antibody (monoclonal mouse anti-rat cytochrome P450 1A1 IgG for experimental section 1, or polyclonal rabbit anti-rat cytochrome P450 1A1 IgG for experimental section 2) was 1 μg/mL (experimental section 1) or 30 μg/mL (experimental section 2), the dilution of secondary antibody (goat anti-mouse IgG for experimental section 1, or goat anti-rabbit IgG for experimental section 2) was 1:3000, and the phosphatase reaction time was 5 min (experimental section 1) or 2 min (experimental section 2). For immunodetection of cytochrome P450 2B, the concentration of primary antibody (backabsorbed polyclonal rabbit anti-rat cytochrome P450 2B1 IgG) was 2.5 μg/mL (experimental sections 1 and 2) or 2 μg/mL (experimental section 3), the dilution of secondary antibody (goal anti-rabbit IgG) was 1:3000, and the phosphatase reaction time ranged from 0.8 to 7.3 min. For immunodetection of cytochrome P450 2E1, the concentration of primary antibody (polyclonal rabbit anti-rat cytochrome P450 2E1 IgG) was approximately 10 μg/mL, the dilution of secondary antibody (goat anti-rabbit IgG) was 1:3000, and the phosphatase reaction time was 1.3 min. For immunodetection of cytochrome P450 3A, the concentration of primary antibody (polyclonal rabbit anti-rat cytochrome P450 3A1 IgG) was 50 μg/mL, the dilution of secondary antibody (goat anti-rabbit IgG) was 1:3000, and the phosphatase reaction time ranged from 1.5 to 3.7 min.

2.11. *IMMUNOQUANTITATION*

Staining intensities of the bands on the nitrocellulose membranes were measured by computer image analysis with a VISAGE 110 Bio Image Analyzer (BioImage, Ann Arbor, MI) consisting of a high resolution camera and a Sun Microsystems Workstation. The amount of immunoreactive protein was determined from the integral of the optical density of the stained band using the Whole Band Analysis software option. Values of the integrated intensity were converted into pmol quantities using calibration curves generated by loading various concentrations of purified cytochrome P450 on gels followed by immunoblotting and densitometric analysis as described above. In addition, a known concentration of purified cytochrome P450 or pooled liver microsomes prepared from rats treated with various inducers, was included on each blot as an internal standard. Calibration curves were prepared by plotting the ratio of the integrated intensity of each authentic standard to the integrated intensity of the internal standard versus the amount of purified cytochrome P450 (i.e. authentic standard) loaded. The pmol quantity corresponding to the stained band for each unknown sample was calculated from the slope of the linear calibration curve using the ratio of the integrated intensity of the immunoreactive sample band to that of the internal standard.

2.12. *DETERMINATION OF METABOLITE COMPLEX FORMATION*

Hepatic microsomes from a control rat (from section 2) were preincubated with 0.1 mM Telazol® in the presence or absence of 1 mM NADPH at 37°C for 20 min, and total cytochrome P450 content was determined spectrally as described above. In addition, total cytochrome P450

content was determined for hepatic microsomes from a rat treated with Telazol® at a dose of 120 mg/kg (from section 2) in the presence or absence of 50 μM potassium ferricyanide.

2.13. *STATISTICAL ANALYSIS*

Results were analyzed by a one way Analysis of Variance (ANOVA) and the differences between pairs of means were tested by the Student-Newman-Keuls test (SNK). In addition, two way Analysis of Variance was used for experimental section 2 to determine the interacting effect between tiletamine and zolazepam. Mean differences that had a p value of < 0.05 were considered to be statistically significant.

3. RESULTS

There were three inter-related sections in this research project, designated as sets 1, 2 and 3. For section 1, the effect of varying doses of Telazol® on hepatic cytochromes P450 in adult male rats was investigated after a single i.p. injection. A single dosing regimen was used to mimic the dose used routinely in the clinic and in the field with various species, which usually involved a single i.m. or i.v. injection instead of repeated daily treatment seen in typical induction studies. The purpose of section 2 was to determine the constituent(s) of Telazol® responsible for the hepatic enzyme induction observed in section 1. In section 3, the inductive effects of Telazol® and its constituent(s) were compared with that produced by PB after a similar dosing regimen. Although PB has been extremely well-studied as a cytochrome P450 inducer, very little information is available regarding its inductive effect after a single injection. Hence, in addition to comparing the pattern of enzyme induction elicited by Telazol® with that elicited by PB, results from the third section will provide new information regarding the relationship between dose and inductive effect of PB following a single treatment.

For each section, the effects of drug treatment on liver and body weight, protein concentration, total hepatic cytochrome P450 content, various cytochrome P450-dependent enzyme activities, and hepatic levels of specific cytochrome P450 isozymes were determined. Results are presented in the following order: 1. the effect of treatment with increasing doses of Telazol® on hepatic cytochrome P450 expression; 2. optimization of BROD and PROD assays; 3. the effects of treatment with Telazol®, tiletamine, or zolazepam on hepatic cytochrome P450 expression; and 4. the effect of a single treatment with PB on hepatic cytochrome P450 expression.

SECTION 1:

3.1. *EFFECTS OF TREATMENT WITH INCREASING DOSES OF TELAZOL® ON BODY WEIGHT AND LIVER WEIGHT IN RATS*

All rats were weighed at the time of treatment and on the day they were killed. The effects of treatment with increasing doses of Telazol® on final body and liver weight are shown in Table 3.1. Results are expressed as mean body weight, mean liver weight, and liver weight as a percentage of body weight. No significant difference was found when body and liver weights of the Telazol®-treated groups were compared to those of the control group, but treatment with Telazol® at a dose of 120 mg/kg produced a significant increase in the liver to body weight ratio. For all other treatment groups, no significant effect on the liver or body weight was observed.

Table 3.1. Effect of treatment with increasing doses of Telazol® on body weight and liver weight in rats.

Dose of Telazol® (mg/kg)	Body weight (g)	Liver weight (g)	Liver weight as a percentage of body weight
Control	291 ± 4	13.1 ± 0.4	4.5 ± 0.1
20	278 ± 8	12.8 ± 0.6	4.6 ± 0.1
40	285 ± 6	12.9 ± 0.6	4.5 ± 0.1
80	283 ± 6	13.5 ± 0.6	4.7 ± 0.1
120	271 ± 5	13.5 ± 0.2	5.0 ± 0.0 †

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

3.2. *EFFECTS OF TREATMENT WITH INCREASING DOSES OF TELAZOL® ON PROTEIN CONCENTRATION AND TOTAL HEPATIC CYTOCHROME P450 CONTENT*

Protein concentration, total cytochrome P450 concentration and total hepatic cytochrome P450 content were determined in hepatic microsomes prepared from control and Telazol®-treated rats and the results are reported in Table 3.2. No significant difference was found with all three parameters between the control and treatment groups.

Table 3.2. Effects of treatment with increasing doses of Telazol® on protein concentration and total hepatic cytochrome P450 content.

Dose of Telazol® (mg/kg)	Protein concentration (mg/mL)	Total cytochrome P450 concentration (nmol/mL)	Total cytochrome P450 content (nmol/mg protein)
Control	22.5 ± 0.7 (1.0)	37.7 ± 2.0 (1.0)	1.7 ± 0.1 (1.0)
20	22.4 ± 2.2 (1.0)	31.0 ± 4.0 (0.8)	1.4 ± 0.1 (0.8)
40	21.6 ± 1.2 (1.0)	34.9 ± 2.7 (0.9)	1.6 ± 0.1 (0.9)
80	25.3 ± 0.6 (1.1)	40.6 ± 1.9 (1.1)	1.6 ± 0.1 (0.9)
120	22.6 ± 0.6 (1.0)	34.5 ± 0.9 (0.9)	1.5 ± 0.0 (0.9)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
There was no significant difference among the group mean values.

3.3. *EFFECTS OF TREATMENT WITH INCREASING DOSES OF TELAZOL® ON BROD, EROD, PROD, AND P-NITROPHENOL HYDROXYLASE ACTIVITIES*

The effects of treatment with varying doses of Telazol® on hepatic microsomal BROD, EROD, PROD and *p*-nitrophenol hydroxylase activities are shown in Table 3.3. Dose-dependent increases in BROD and PROD activities were observed. BROD activity was increased by approximately seven-fold in hepatic microsomes from rats treated with Telazol® at doses of 80 mg/kg and greater. A small but significant increase in EROD activity was found in hepatic microsomes from rats treated with Telazol® at doses of 40 and 120 mg/kg, and an increase of approximately three-fold in PROD activity was produced by drug treatment at doses of 80 mg/kg and greater. No significant difference was found in *p*-nitrophenol hydroxylase activity between control and treatment groups at all doses tested.

3.4. *EFFECTS OF TREATMENT WITH INCREASING DOSES OF TELAZOL® ON TESTOSTERONE HYDROXYLASE ACTIVITIES*

Table 3.4 indicates the effects of treatment with increasing doses of Telazol® on testosterone hydroxylase activities. A significant decrease was seen in testosterone 2α- and 16α-hydroxylase activities in hepatic microsomes from rats treated with the highest doses of Telazol®. No significant difference was found in the rate of 6β-, 7α-hydroxytestosterone , or androstenedione formation between control and treatment groups. In contrast, there was a dose-dependent increase in testosterone 16β-hydroxylase activity. Treatment with Telazol® at a dose of 120 mg/kg produced an approximately six-fold increase in testosterone 16β-hydroxylase activity.

Table 3.3. Effects of increasing doses of Telazol® on BROD, EROD, PROD and *p*-nitrophenol hydroxylase activities.

Dose of Telazol® (mg/kg)	BROD activity (pmol/mg/min)	EROD activity (pmol/mg/min)	PROD activity (pmol/mg/min)	*p*-Nitrophenol hydroxylase activity (nmol/mg/min)
Control	539 ± 45 (1.0)	740 ± 27 (1.0)	600 ± 131 (1.0)	1.1 ± 0.1 (1.0)
20	1473 ± 180 (2.7)	729 ± 101 (1.0)	730 ± 129 (1.2)	1.2 ± 0.1 (1.1)
40	2243 ± 362 † (4.2)	1015 ± 47 † (1.4)	1226 ± 297 (2.0)	1.2 ± 0.1 (1.1)
80	3831 ± 383 † (7.1)	887 ± 26 (1.2)	1512 ± 150 † (2.5)	1.2 ± 0.1 (1.1)
120	3876 ± 540 † (7.2)	1142 ± 48 † (1.5)	1732 ± 165 † (2.9)	1.4 ± 0.1 (1.3)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

Table 3.4. Effects of treatment with increasing doses of Telazol® on testosterone hydroxylase activities.

Dose of Telazol® (mg/kg)	Testosterone metabolites (pmol metabolite formed/min/mg protein)					
	2α	6β	7α	16α	16β	androstenedione
control	3752 ± 165 (1.0)	2174 ± 185 (1.0)	228 ± 13 (1.0)	3465 ± 177 (1.0)	68 ± 6 (1.0)	909 ± 43 (1.0)
20	2899 ± 377 † (0.8)	2219 ± 315 (1.0)	242 ± 77 (1.1)	2890 ± 381 (0.8)	158 ± 14 (2.3)	862 ± 87 (0.9)
40	2902 ± 166 † (0.8)	2634 ± 167 (1.2)	312 ± 33 (1.4)	2942 ± 138 (0.8)	222 ± 30 † (3.3)	1002 ± 133 (1.1)
80	2133 ± 178 † (0.6)	2290 ± 228 (1.1)	301 ± 19 (1.3)	2373 ± 161 † (0.7)	342 ± 36 † (5.0)	845 ± 55 (0.9)
120	2043 ± 171 † (0.5)	2488 ± 219 (1.1)	287 ± 21 (1.3)	2355 ± 241 † (0.7)	388 ± 63 † (5.7)	893 ± 61 (1.0)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

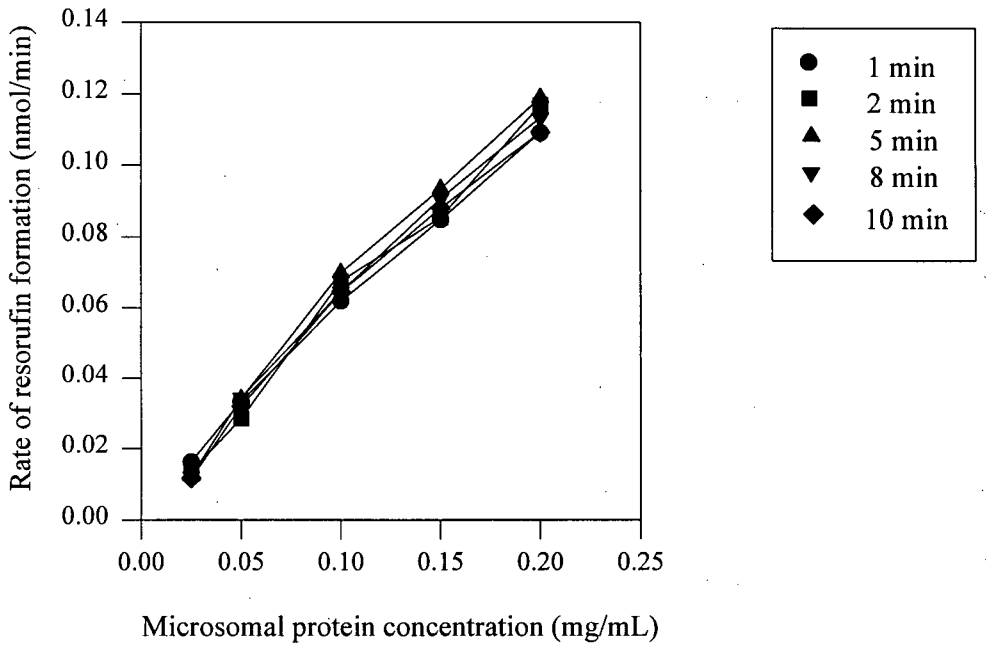
3.5. *CONDITION OPTIMIZATION FOR BROD AND PROD ASSAYS*

Unexpectedly high values of microsomal BROD and PROD activities for untreated rats were obtained in the section in which varying doses of Telazol® were examined. BROD and PROD activities of hepatic microsomes from untreated rats were five-fold and more than 30-fold greater, respectively, than values typically reported in the literature (146). To ensure that each enzyme assay was conducted under optimal and reproducible conditions, assay conditions were re-examined. Previously prepared, pooled hepatic microsomes from untreated and PB-treated rats were used for each assay, and the results are shown below.

3.5.1. *Effect of microsomal protein concentration on BROD activity*

The effect of varying microsomal protein concentration on BROD activity was determined. Figure 3.1 shows the effect of varying protein concentration on rate of resorufin formation with hepatic microsomes from untreated and PB-treated rats at various reaction times. Enzyme activity was greatest at a microsomal protein concentration within the range of 0.025 to 0.1 mg/mL of reaction mixture for hepatic microsomes from untreated rats, and enzyme activity was reduced slightly at a protein concentration between 0.1 and 0.2 mg/mL. With hepatic microsomes from PB-treated rats, the rate of resorufin formation was linearly related to protein concentration up to a concentration of 0.05 mg/mL only with reaction times of 2 min or less, and linearity declined starting at a protein concentration of 0.05 mg/mL for reactions that proceeded for 5 min or longer. For both untreated and treated samples, a microsomal protein concentration in the range of 0.025 to 0.05 mg/mL produced easily

(A)



(B)

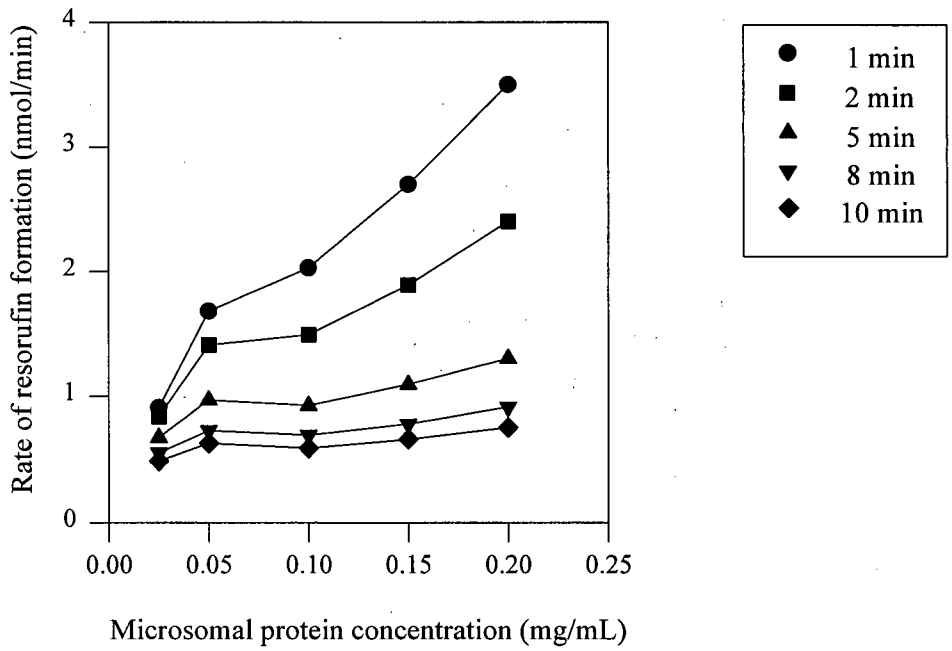
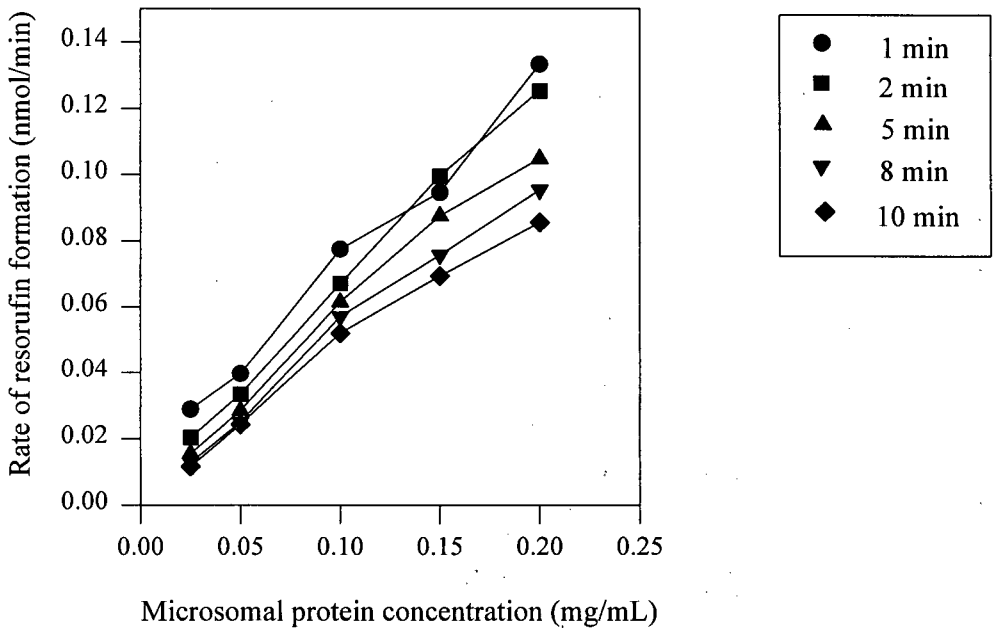


Figure 3.1. Effect of microsomal protein concentration on BROD activity in hepatic microsomes prepared from (A) untreated rats and (B) rats treated with phenobarbital.

(A)



(B)

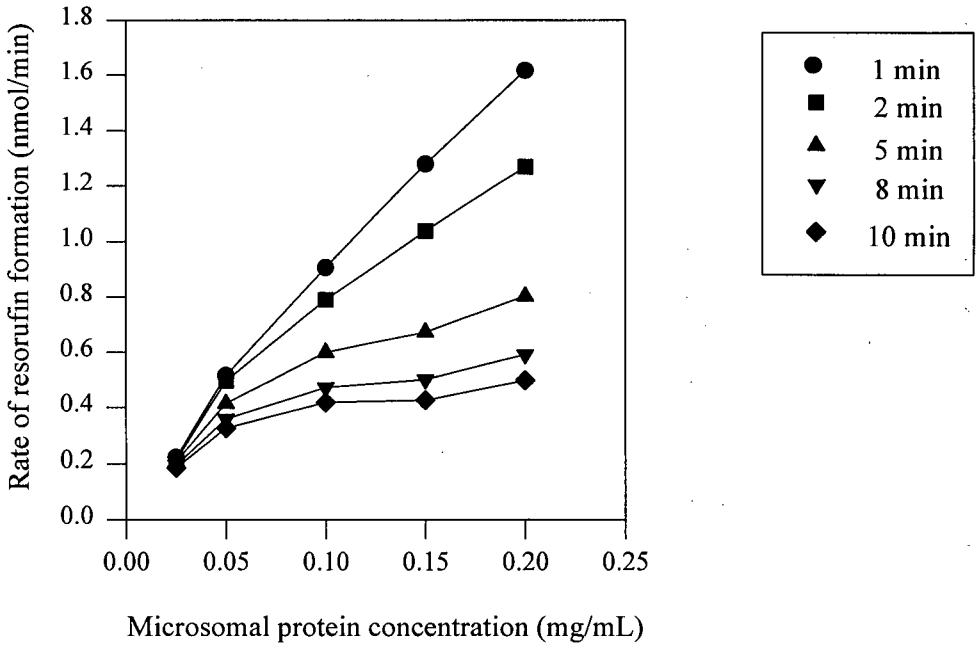


Figure 3.2. Effect of microsomal protein concentration on PROD activity in hepatic microsomes prepared from (A) untreated rats and (B) rats treated with phenobarbital.

measureable and reproducible enzyme activities for reactions that proceeded for a period of 1 to 2 min.

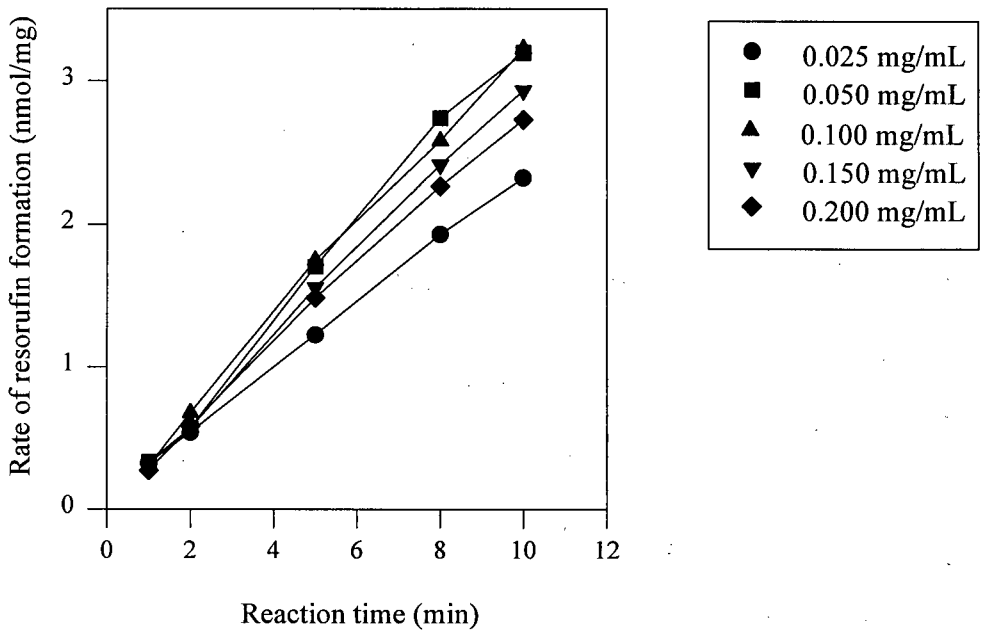
3.5.2. *Effect of microsomal protein concentration on PROD activity*

The effect of microsomal protein concentration on PROD activity in hepatic microsomes from untreated and PB-treated rats is shown in Figure 3.2. Similar to the BROD assay, PROD activity was linearly related to protein concentration, in the range tested (0.025-0.2 mg/mL), with liver microsomes from untreated rats for reaction times of 2 min or less. With hepatic microsomes from PB-treated rats, the rate of product formation was greatest at a protein concentration of 0.025 to 0.05 mg/mL with a reaction time of 1 to 2 min. Activity was decreased for reactions that proceeded for 5 min and longer, especially at protein concentrations greater than 0.05 mg/mL. The results suggested that a microsomal protein concentration within the range of 0.025 to 0.2 mg/mL could be used only for a reaction period of 1 to 2 min with liver microsomes from PB-induced rats.

3.5.3. *Effect of reaction time on BROD activity*

The same data illustrated in Figure 3.1 was re-plotted to show the effect of reaction time, which ranged from 1 to 10 min, on BROD activity in hepatic microsomes from untreated and PB-treated rats. Regardless of the microsomal protein concentration, the rate of resorufin formation in hepatic microsomes from untreated rats increased linearly with reaction time up to 10 min. For hepatic microsomes prepared from PB-treated rats, BROD activity measured at a microsomal protein concentration of 0.025 and 0.05 mg/mL increased linearly with reaction

(A)



(B)

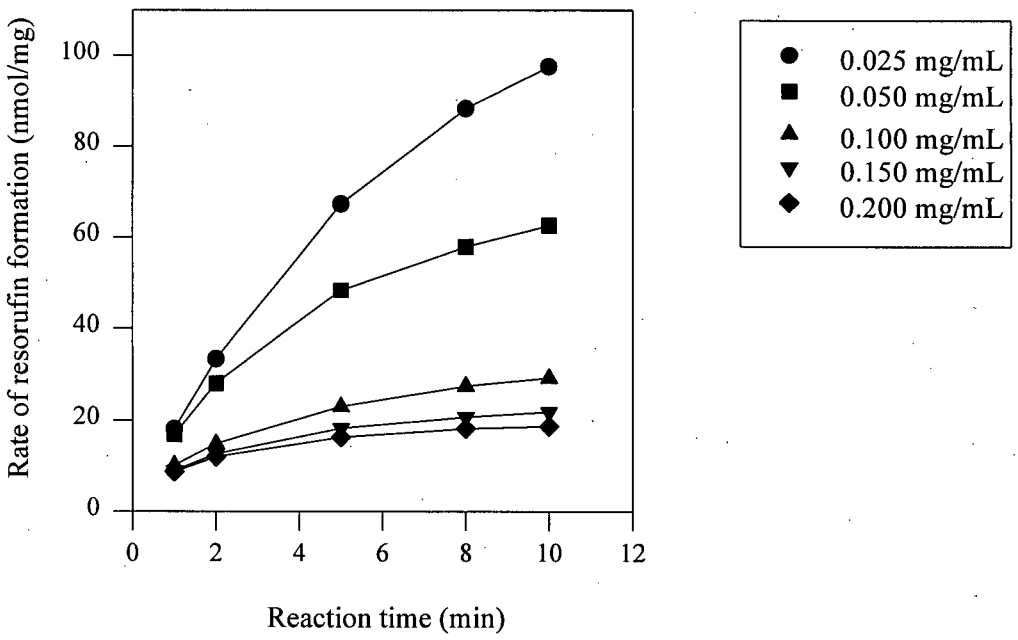
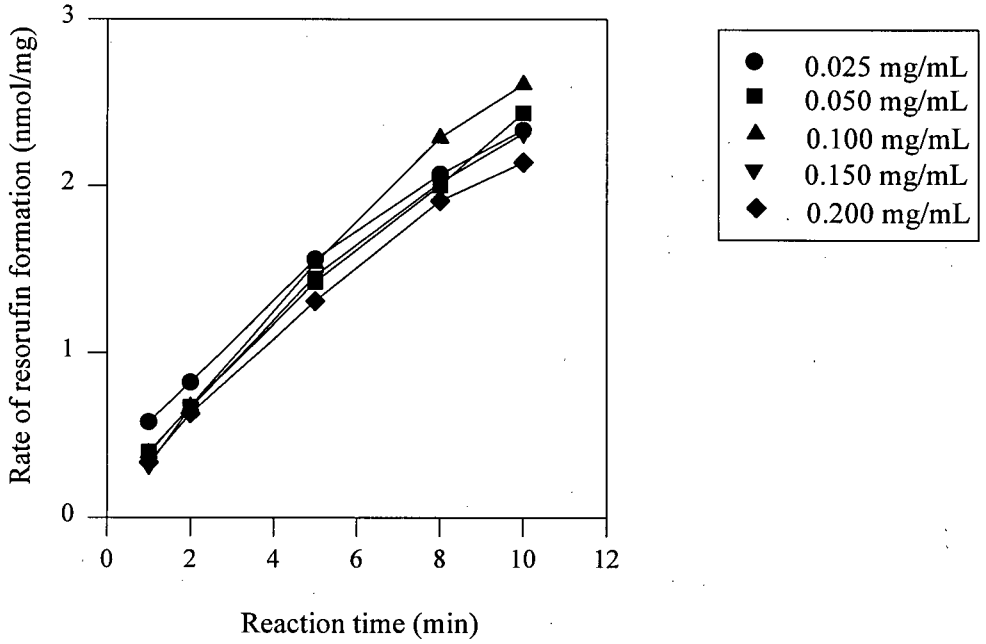


Figure 3.3. Effect of reaction time on BROD activity in hepatic microsomes prepared from (A) untreated rats and (B) rats treated with phenobarbital.

(A)



(B)

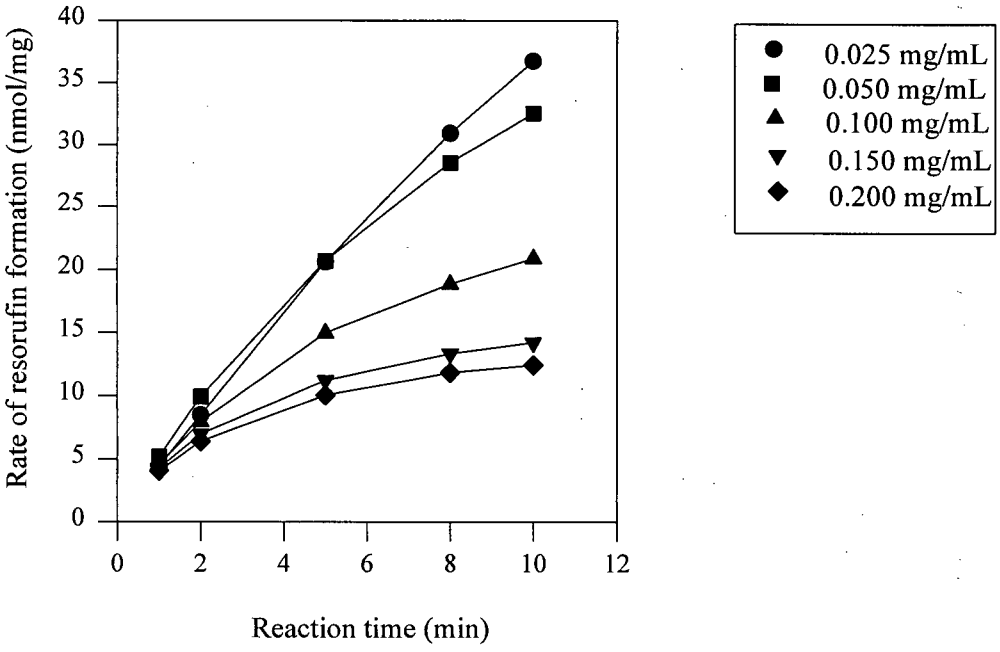


Figure 3.4. Effect of reaction time on PROD activity in hepatic microsomes prepared from (A) untreated rats and (B) rats treated with phenobarbital.

time up to 2 min before starting to level off. Poor linearity between the rate of resorufin formation and reaction time for samples that had a protein concentration of 0.1 mg/mL or greater was observed.

3.5.4. *Effect of reaction time on PROD activity*

For liver microsomes from untreated rats, the rate of resorufin formation increased proportionally with reaction time for all microsomal protein concentrations tested, indicating a reaction time within the range of 1 to 8 min was optimal for measurement of PROD activity (Figure 3.4.A). For hepatic microsomes from rats treated with PB, the rate of resorufin formation increased linearly with reaction time up to 5 min at a protein concentration of 0.025 to 0.05 mg/mL (Figure 3.4.B). At protein concentrations of 0.1 to 0.2 mg/mL, enzyme activity was maximal at a reaction time of 1 min or less.

3.5.5. *Summary of condition optimization for BROD and PROD assays*

In summary, use of a microsomal protein concentration of 0.05 mg/mL and a reaction time of 2 min were optimal for measurement of BROD and PROD activities in hepatic microsomes from untreated and PB-treated rats. Hence, enzyme assays were conducted under these conditions for all experiments described in section 1. For sections 2 and 3, BROD and PROD assays were performed at a protein concentration of 0.05 mg/mL and a reaction time of 2 min or 5 min. Because enzyme activities measured under these two conditions were similar, only those obtained from a 5-min reaction time were reported for the two sections.

3.6. *IMMUNOBLOT ANALYSIS OF HEPATIC MICROSOMES FROM RATS TREATED WITH INCREASING DOSES OF TELAZOL®*

To confirm that cytochrome P450 2B1 and cytochrome P450 2B2 were selectively induced by treatment with Telazol®, hepatic microsomal samples were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and probed with antibodies against rat cytochrome P450 1A1 and cytochrome P450 2B1. In addition, hepatic levels of cytochrome P450 2B isozymes were subsequently determined by densitometric analysis. Known concentrations of purified cytochrome P450 isozymes were included on each blot as internal standards. The specificity of each antibody had been assessed previously using enzyme-linked immunosorbent and immunoblot assays with purified rat cytochrome P450 isozymes and with different rat liver microsomal preparations.

3.6.1. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 1A1 IgG*

Immunoblots containing microsomal samples prepared from rats treated with Telazol® at a dose of 20, 40, 80 , or 120 mg/kg were probed with monoclonal anti-cytochrome P450 1A1 IgG. This antibody recognizes rat cytochrome P450 1A1 and cytochrome P450 1A2, but does not react with other cytochrome P450 isozymes. Various concentrations of purified cytochrome P450 1A1 were also included on the immunoblot (Figure 3.5). The upper band in each lane represents cytochrome P450 1A1, and the lower band represents cytochrome P450 1A2. Very little or no cytochrome P450 1A1 was found in lanes containing hepatic microsomes from untreated rats or rats treated with Telazol®, while a darkly stained band

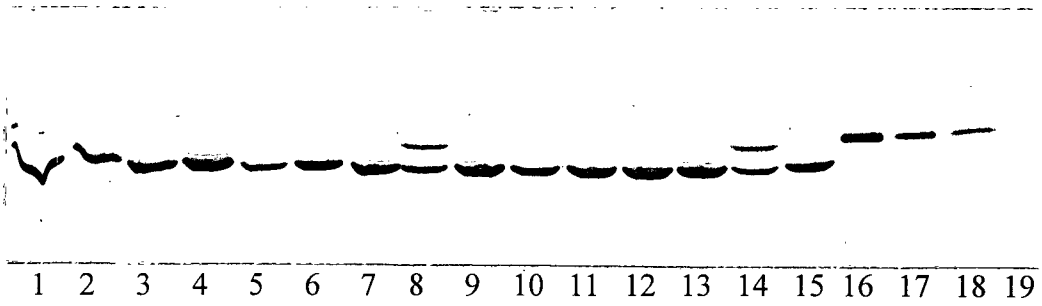


Figure 3.5. Immunoblot of rat hepatic microsomes probed with monoclonal mouse antibody against rat cytochrome P450 1A1. Final concentration of microsomal protein was 10 μg per lane. Concentration of primary antibody (mouse anti-cytochrome P450 1A1) was 1 μg/mL. Dilution of secondary antibody (goat anti-mouse IgG) was 1:3000. Alkaline phosphatase reaction time was 5 min. Lanes 1 to 3 contain microsomes from vehicle-treated rats, lanes 4 to 15 contain microsomes from rats treated with Telazol® at a dose of 20 mg/kg (lanes 4 to 6), 40 mg/kg (lanes 7 to 9), 80 mg/kg (lanes 10 to 12), or 120 mg/kg (lanes 13 to 15). Lanes 16 to 19 contain purified rat cytochrome P450 1A1 at a concentration of 0.2, 0.1, 0.05, or 0.025 pmol per lane, respectively.

Table 3.5. Effects of treatment with increasing doses of Telazol® on hepatic levels of cytochrome P450 1A1 and cytochrome P450 1A2.

Dose of Telazol® (mg/kg)	Amount of immunoreactive cytochrome P450 1A1 protein*	Amount of immunoreactive cytochrome P450 1A2 protein
control	nd	586.2 ± 181.9 (1.0)
20	nd	657.1 ± 175.1 (1.1)
40	nd	546.3 ± 98.9 (0.9)
80	nd	635.6 ± 94.8 (1.1)
120	nd	362.2 ± 88.8 (0.6)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Each value was expressed as the integrated intensity of the band per milligram protein.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
* The hepatic level of immunoreactive cytochrome P450 1A1 protein was not determined (nd) because a distinct protein band corresponding to this isozyme could be detected in only one sample from each group.
There was no significant difference among the group mean values.

corresponding to cytochrome P450 1A2 was observed in all the lanes that contained samples from both untreated and treatment groups.

Results of immunoquantitation of cytochrome P450 1A isozymes are displayed in Table 3.5. Because only one sample from each group showed a distinct band of cytochrome P450 1A1, the mean hepatic level of the isozyme in each group was not determined. A standard curve could not be generated for cytochrome P450 1A2 because of a lack of purified cytochrome P450 1A2. Hence, the hepatic level of this isozyme is given as integrated intensity per milligram of microsomal protein. Results of immunoquantitation of cytochrome P450 1A2 indicated no significant difference in the integrated intensity of bands corresponding to cytochrome P450 1A2 between treatment and control groups.

3.6.2. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 2B1 IgG*

Figure 3.6 shows an immunoblot of hepatic microsomes from untreated and Telazol®-treated rats probed with polyclonal anti-rat cytochrome P450 2B1 IgG. Two prominent immunostained bands were visible in each lane. The two bands represent cytochrome P450 2B1 (lower band) and cytochrome P450 2B2 (upper band). The antibody also reacted with a third related protein, cytochrome P450 2B3, which corresponded to the weakly stained band found below the cytochrome P450 2B1 band in all the lanes. Staining intensity of the immunoreactive band corresponding to cytochrome P450 2B1 appeared to be enhanced proportionally with an increase in drug dosage for samples from Telazol®-treated rats.

Hepatic levels of cytochrome P450 2B isozymes were determined by densitometric analysis and the results are displayed in Table 3.6. Results are expressed as cytochrome P450

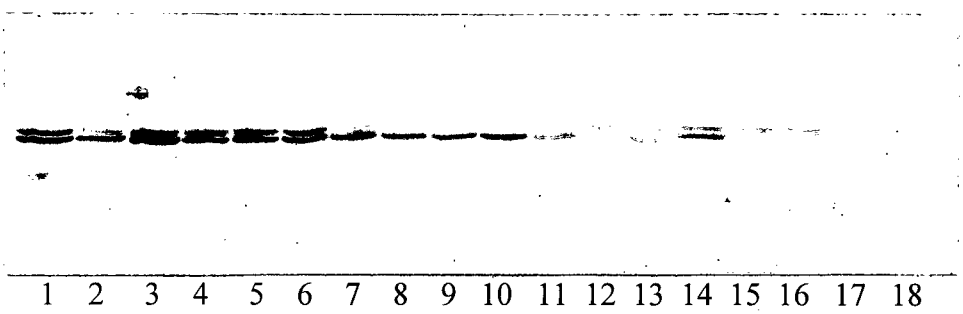


Figure 3.6. Immunoblot of rat hepatic microsomes probed with polyclonal antibody against rat cytochrome P450 2B1. Final concentration of microsomal samples was 10 μg microsomal protein per lane. Concentration of primary antibody (rabbit anti-cytochrome P450 2B1 IgG) was 2.5 μg/mL. Dilution of secondary antibody (goat anti-rabbit IgG) was 1:3000. Alkaline phosphatase reaction time was 5.3 min. Lanes 1 to 14 contain microsomes from rats treated with Telazol® at a dose of 120 mg/kg (lanes 1 to 3), 80 mg/kg (lanes 4 to 7), 40 mg/kg (lanes 8 to 10), or 20 mg/kg (lanes 11 to 14). Lanes 15 to 18 contain microsomes from vehicle-treated rats.

Table 3.6. Effects of treatment with increasing doses of Telazol® on hepatic levels of cytochrome P450 2B1 and cytochrome P450 2B2.

Dose of Telazol® (mg/kg)	Cytochrome P450 2B1 content (pmol/mg protein)	Cytochrome P450 2B1 as percentage of total cytochrome P450	Cytochrome P450 2B2 content (pmol/mg protein)	Cytochrome P450 2B2 as percentage of total cytochrome P450
control	2.2 ± 0.8 (1.0)	0.1 ± 0.1 (1.0)	8.2 ± 1.4 (1.0)	0.5 ± 0.1 (1.0)
20	11.3 ± 2.5 (5.1)	0.9 ± 0.2 (9.0)	10.8 ± 2.1 (1.3)	0.8 ± 0.1 (1.6)
40	20.7 ± 3.1 † (9.4)	1.3 ± 0.2 † (13.0)	10.6 ± 0.8 (1.3)	0.7 ± 0.1 (1.4)
80	22.2 ± 3.0 † (10.1)	1.4 ± 0.2 † (14.0)	12.1 ± 1.1 (1.5)	0.8 ± 0.1 (1.6)
120	37.7 ± 6.7 † (17.1)	2.5 ± 0.4 † (25.0)	17.9 ± 5.2 (2.2)	1.2 ± 0.3 (2.4)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

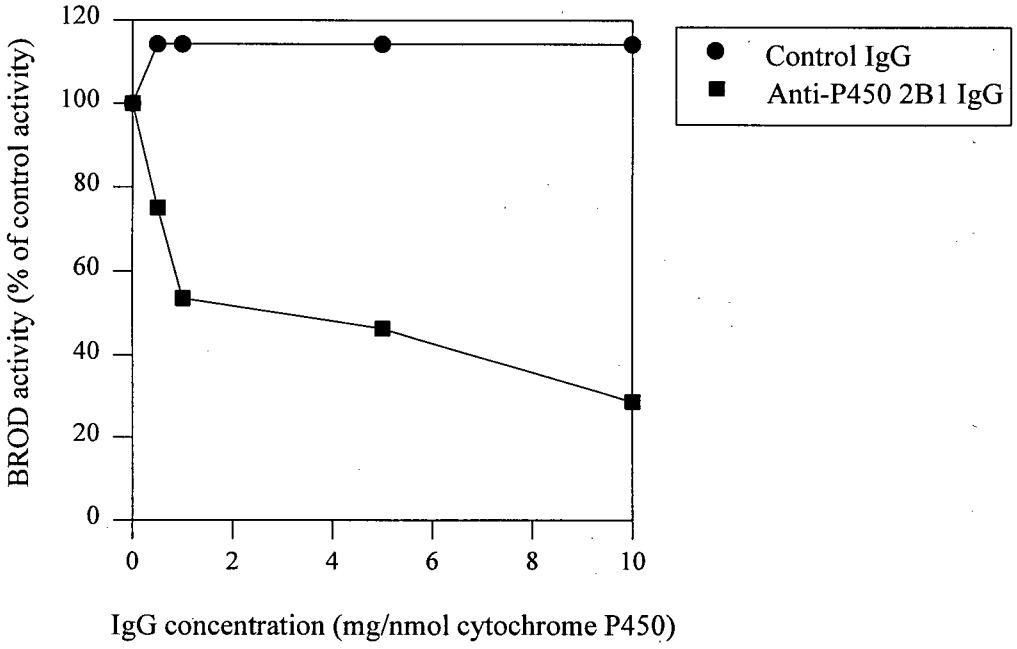
2B content and cytochrome P450 2B content as a percentage of total cytochrome P450. Various amounts of purified cytochrome P450 2B1 were incubated on the gel for construction of a standard curve. Treatment with Telazol® produced a dose-dependent increase in levels of the enzyme. A significant increase of approximately 17-fold was found in hepatic level of cytochrome P450 2B1 for rats treated with Telazol® at a dose of 120 mg/kg, and an approximately two-fold increase was seen in the hepatic level of cytochrome P450 2B2, however, this increase was not statistically significant.

3.7. *ANTIBODY INHIBITION STUDIES*

In order to verify the catalytic role of cytochrome P450 2B isozymes in BROD and PROD activities of hepatic microsomes prepared from rats treated with varying doses of Telazol®, antibody inhibition studies were performed using polyclonal antibody against cytochrome P450 2B1. The antibody recognized cytochrome P450 2B1, cytochrome P450 2B2 and a third, noninducible form of cytochrome P450 2B subfamily. Two microsomal samples from the control group and two samples from the group treated with Telazol® at a dose of 120 mg/kg were tested for each assay. Because samples from the same treatment group displayed similar enzyme activities, results from only one sample from each group is shown.

The effect of incubating hepatic microsomes from a control rat and a rat treated with Telazol® at a dose of 120 mg/kg with increasing concentrations of antibody to cytochrome P450 2B on BROD activity is represented by Figure 3.7. In the presence of 0.5 mg of anti-cytochrome P450 2B1 IgG/nmol cytochrome P450, BROD activities of hepatic microsomes from the control and Telazol®-treated rats were inhibited by approximately 25% and 92%,

(A)



(B)

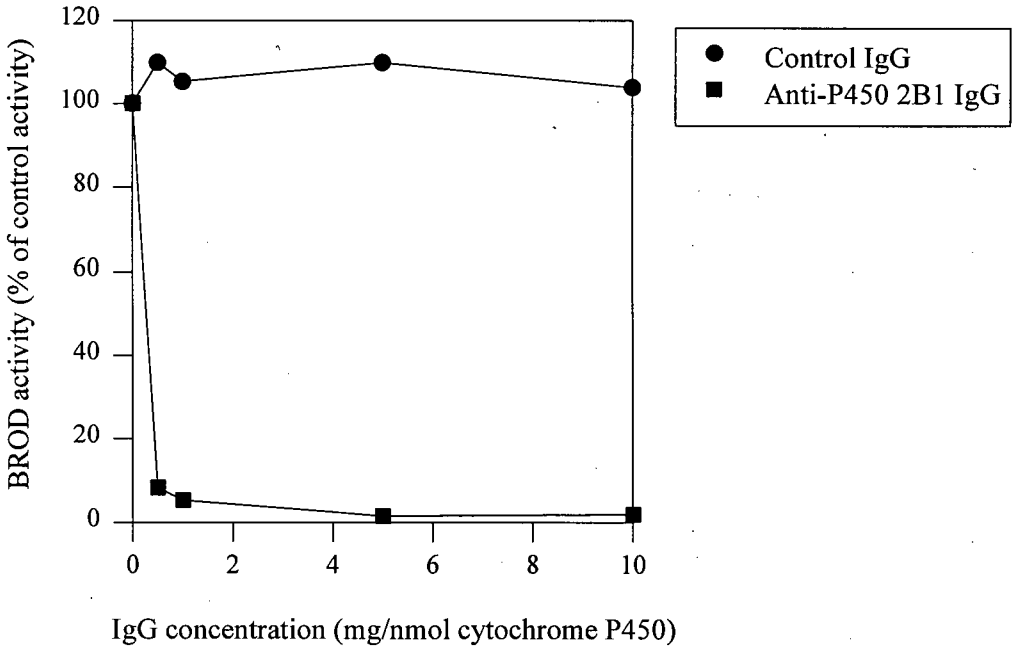
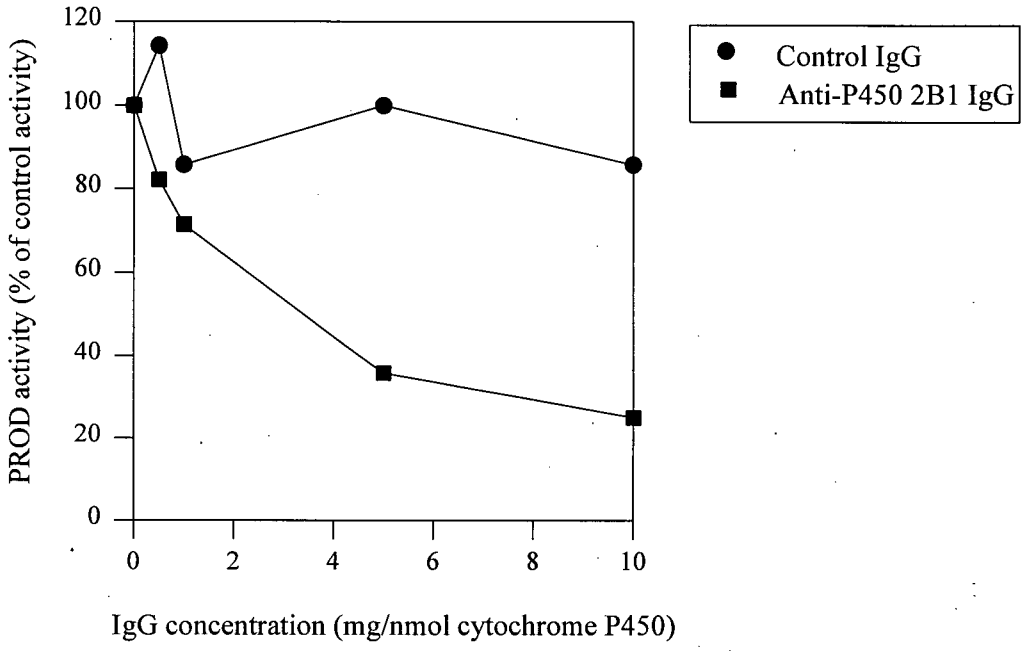


Figure 3.7. Effect of polyclonal anti-cytochrome P450 2B1 IgG on BROD activity in hepatic microsomes prepared from a rat treated with (A) vehicle and (B) Telazol at a dose of 120 mg/kg.

(A)



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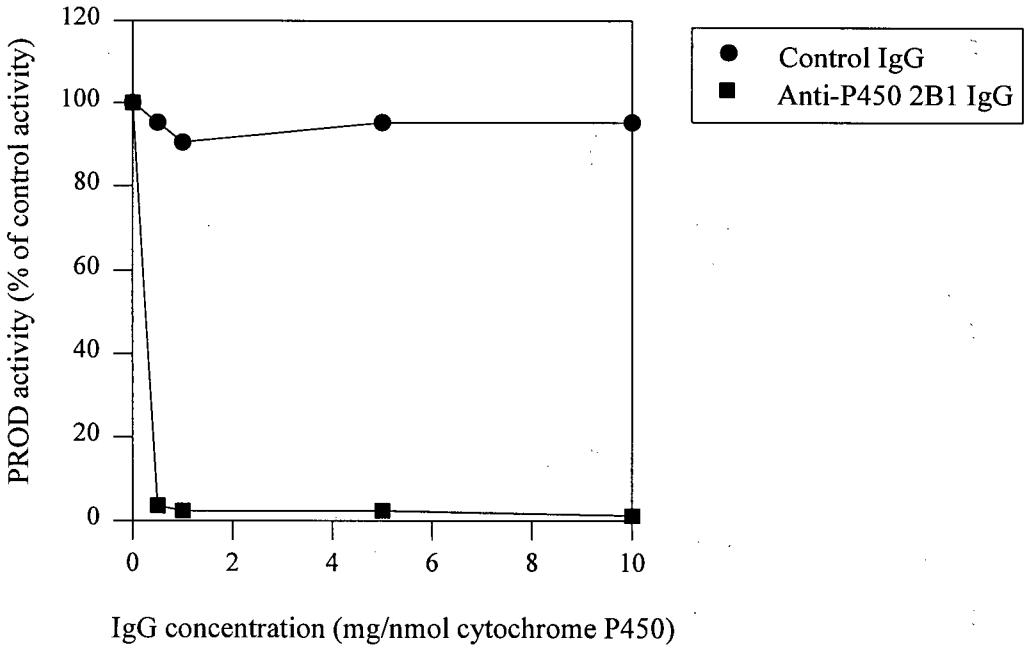

Figure 3.8. Effect of polyclonal anti-cytochrome P450 2B1 IgG on PROD activity in hepatic microsomes prepared from a rat treated with (A) vehicle and (B) Telazol at a dose of 120 mg/kg.

respectively, whereas in the presence of 5 mg anti-cytochrome P450 2B1 IgG/nmol cytochrome P450, the enzyme activities of hepatic microsomes from the same control and treated rat were inhibited by approximately 55% and greater than 95%, respectively. No enzyme inhibition was observed in the presence of control IgG.

The effect of incubating hepatic microsomes from a control and a rat treated with Telazol® at a dose of 120 mg/kg with increasing concentrations of antibody to cytochrome P450 2B on PROD activity is shown in Figure 3.8. PROD activities of hepatic microsomes from a control and Telazol®-treated rat were inhibited by approximately 18% and 96%, respectively, in the presence of 0.5 mg anti-cytochrome P450 2B1 IgG/nmol cytochrome P450, and by approximately 64% and 98%, respectively, in the presence of 5 mg anti-cytochrome P450 2B1 IgG/nmol cytochrome P450. No inhibition of PROD activity was observed in the presence of control IgG.

SECTION 2:

Previous results indicated that cytochrome P450 expression was induced by treatment with Telazol® at a dose of 120 mg/kg, which was equivalent to a dose of 60 mg/kg of tiletamine and 60 mg/kg of zolazepam. Hence for this section, single i.p. doses of 60 mg/kg of tiletamine and zolazepam were used in order to determine the effect of each constituent on enzyme expression. In addition, the effects of tiletamine and zolazepam on cytochrome P450 induction were compared with those produced by a single i.p. treatment with Telazol® at a dose of 120 mg/kg in the same study.

3.8. *EFFECTS OF TREATMENT WITH TELAZOL®, TILETAMINE, AND ZOLAZEPAM ON BODY WEIGHT AND LIVER WEIGHT IN RATS*

Mean body and liver weight of rats treated with Telazol®, tiletamine, or zolazepam are shown in Table 3.7. The results indicated that all three agents had no significant effect on body or liver weight in rats.

3.9. *EFFECTS OF TREATMENT WITH TELAZOL®, TILETAMINE, AND ZOLAZEPAM ON PROTEIN CONCENTRATION AND TOTAL HEPATIC CYTOCHROME P450 CONTENT*

Total cytochrome P450 contents of hepatic microsomes prepared from control rats and rats treated with Telazol® , tiletamine, or zolazepam are shown in Table 3.8. Results indicated that treatment with each of the three agents had no signficant effect on protein concentration, total hepatic cytochrome P450 concentration, or total hepatic cytochrome P450 content.

Table 3.7. Effects of treatment with Telazol®, tiletamine, and zolazepam on body weight and liver weight in rats.

Treatment (Dose)	Body weight (g)	Liver weight (g)	Liver weight as a percentage of body weight
Control	288 ± 8	13.4 ± 0.3	4.7 ± 0.2
Telazol® (120 mg/kg)	277 ± 7	13.8 ± 0.6	5.0 ± 0.2
Tiletamine (60 mg/kg)	280 ± 4	12.9 ± 0.5	4.6 ± 0.1
Zolazepam (60 mg/kg)	286 ± 7	13.0 ± 0.4	4.5± 0.1

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
There was no significant difference among the group mean values.

Table 3.8. Effects of treatment with Telazol®, tiletamine, and zolazepam on protein concentration and total hepatic cytochrome P450 content.

Treatment (Dose)	Protein concentration (mg/mL)	Total cytochrome P450 concentration (nmol/mL)	Total cytochrome P450 content (nmol/mg protein)
Control	29.3 ± 1.5 (1.0)	40.1 ± 3.8 (1.0)	1.4 ± 0.1 (1.0)
Telazol® (120 mg/kg)	25.4 ± 1.9 (0.9)	31.3 ± 2.4 (0.8)	1.2 ± 0.0 (0.9)
Tiletamine (60 mg/kg)	25.1 ± 1.0 (0.9)	32.4 ± 2.4 (0.8)	1.3 ± 0.1 (0.9)
Zolazepam (60 mg/kg)	25.6 ± 1.4 (0.9)	31.8 ± 1.5 (0.8)	1.2 ± 0.1 (0.9)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
There was no significant difference among the group mean values.

3.10. *EFFECTS OF TREATMENT WITH TELAZOL®, TILETAMINE, AND ZOLAZEPAM ON BROD , EROD AND PROD ACTIVITIES*

Effects of treatment with Telazol®, tiletamine, or zolazepam on BROD, EROD, and PROD activities are summarized in Table 3.9. Treatment with tiletamine produced a small and insignificant increase in BROD activity, while treatment with Telazol® and zolazepam resulted in significant increases of 15- and 9-fold, respectively. All three agents had no effect on EROD activity. Treatment with Telazol® increased PROD activity by 4.5-fold, whereas no significant increase was seen with tiletamine or zolazepam treatment.

3.11. *EFFECTS OF TREATMENT WITH TELAZOL®, TILETAMINE, AND ZOLAZEPAM ON TESTOSTERONE HYDROXYLASE ACTIVITIES.*

The effects of treatment with Telazol®, tiletamine, or zolazepam on testosterone hydroxylation are shown in Table 3.10. Treatment with all three agents produced a significant decrease in testosterone 2α- and 16α-hydroxylation relative to the control group, while no significant change in testosterone 6β- or 7α-hydroxylation was found. Treatment with tiletamine resulted in a small and insignificant increase in testosterone 16β-hydroxylase activity, whereas treatment with Telazol® and zolazepam produced increases of eight- and five-fold, respectively. No significant difference in the rate of androstenedione formation was seen between the control and treatment groups.

Table 3.9. Effects of treatment with Telazol®, tiletamine, and zolazepam on BROD, EROD, and PROD activities.

Treatment (Dose)	BROD activity (pmol/mg/min)	EROD activity (pmol/mg/min)	PROD activity (pmol/mg/min)
Control	257 ± 24 (1.0)	409 ± 44 (1.0)	255 ± 23 (1.0)
Telazol® (120 mg/kg)	3933 ± 634 † (15.3)	429 ± 70 (1.0)	1139 ± 222 † (4.5)
Tiletamine (60 mg/kg)	536 ± 66 (2.1)	441 ± 75 (1.1)	309 ± 75 (1.2)
Zolazepam (60 mg/kg)	2292 ± 201 † (8.9)	438 ± 70 (1.1)	707 ± 93 (2.8)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

Table 3.10. Effects of treatment with Telazol®, tiletamine, and zolazepam on testosterone hydroxylase activities.

Treatment (Dose)	Testosterone metabolites (pmol metabolite formed/min/mg protein)					
	2α	6β	7α	16α	16β	androstenedione
Control	2628 ± 316 (1.0)	2521 ± 242 (1.0)	204 ± 21 (1.0)	3553 ± 426 (1.0)	192 ± 15 (1.0)	2354 ± 322 (1.0)
Telazol® (120 mg/kg)	903 ± 150 † (0.3)	2534 ± 212 (1.0)	241 ± 34 (1.2)	2067 ± 198 † (0.6)	1522 ± 271 † (7.9)	2486 ± 299 (1.1)
Tiletamine (60 mg/kg)	1753 ± 215 † (0.7)	2278 ± 226 (0.9)	218 ± 18 (1.1)	2475 ± 308 † (0.7)	284 ± 32 (1.5)	1847 ± 194 (0.8)
Zolazepam (60 mg/kg)	1315 ± 100 † (0.5)	2698 ± 248 (1.1)	256 ± 18 (1.3)	2317 ± 208 † (0.7)	895 ± 58 † (4.7)	1996 ± 194 (0.8)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

3.12. *IMMUNOBLOT ANALYSIS OF HEPATIC MICROSOMES PREPARED FROM RATS TREATED WITH TELAZOL®, TILETAMINE, OR ZOLAZEPAM*

To determine if treatment with tiletamine or zolazepam had any effect on hepatic levels of cytochrome P450 1A, cytochrome P450 2B, cytochrome P450 2E, and cytochrome P450 3A isozymes, immunoblots containing microsomal proteins from control and treatment groups were prepared and probed with polyclonal antibodies against various forms of rat cytochromes P450. Fixed concentrations of purified cytochrome P450 isozymes or pooled liver microsomes from rats treated with specific inducing agents were included on each blot was internal standards. Densitometric quantitation was performed on each immunoblot and the results of qualitative and quantitative analysis are presented below.

3.12.1. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 1A1 IgG*

An immunoblot containing hepatic microsomes from untreated rats and rats treated with Telazol®, tiletamine, or zolazepam was probed with anti-cytochrome P450 1A1 IgG (results not shown). Darkly stained bands were visible in lanes that contained purified cytochrome P450 1A1 and microsomes from rats treated with 3-MC. Staining was very weak in all other lanes containing hepatic microsomes from control and Telazol®-, tiletamine-, and zolazepm-treated rats. Immunoquantitation of this blot was not conducted because of the weak immunoreactive staining and because there appeared to be no difference in staining between lanes containing untreated and treated liver microsomes.

3.12.2. *Immunoblot analysis of hepatic microsomes probed with cytochrome P450 2B1 IgG*

An immunoblot containing hepatic microsomes from untreated rats and rats treated with Telazol®, tiletamine, or zolazepam was probed with anti-rat cytochrome P450 2B1 IgG, and is shown in Figure 3.9. A standard curve was constructed from various concentrations of purified cytochrome P450 2B1. The staining intensity of individual bands increased progressively from lanes containing samples prepared from untreated rats, to rats treated with tiletamine, zolazepam, and Telazol®. The intensity of the band corresponding to cytochrome P450 2B1 (middle band) increased to a much greater extent than that of the band corresponding to cytochrome P450 2B2 (upper band) after drug treatment. Hepatic levels of cytochrome P450 2B1 and cytochrome P450 2B2 were quantitated by densitometry and are shown below. A third, non-inducible form of the subfamily, cytochrome P450 2B3, could be seen in some lanes that contained hepatic microsomes of untreated, tiletamine-treated, or zolazepam-treated rats.

Results of immunoquantitation of cytochrome P450 2B1 and cytochrome P450 2B2 in hepatic microsomes from rats treated with Telazol®, tiletamine, or zolazepam are displayed in Table 3.11. Treatment with Telazol® and zolazepam increased the hepatic level of cytochrome P450 2B1 by 73- and 44-fold, respectively, while the same treatment elevated the hepatic level of cytochrome P450 2B2 by nine- and seven-fold, respectively. The extent of increase in both cytochrome P450 2B isozymes was much greater than that observed in the previous section (Table 3.5). Treatment with tiletamine also caused an increase in the hepatic content of both isozymes, however, the increase was not statistically significant.

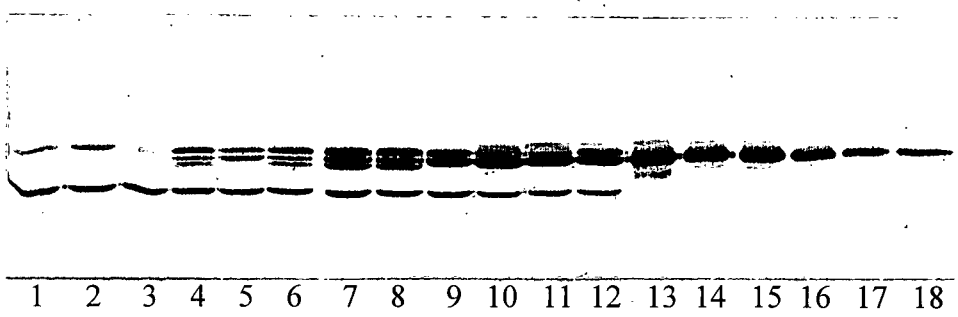

Figure 3.9. Immunoblot of rat hepatic microsomes probed with polyclonal antibody against rat cytochrome P450 2B1. Final concentration of microsomal samples was 10 μg microsomal protein per lane. Concentration of primary antibody (rabbit anti-cytochrome P450 2B1 IgG) was 2.5 μg/mL. Dilution of secondary antibody (goat anti-rabbit IgG) was 1:3000. Alkaline phosphatase reaction time was 2 min. Lanes 1 to 3 contain microsomes from vehicle-treated rats. Lanes 4 to 6 contain microsomes from rats treated with tiletamine at a dose of 60 mg/kg. Lanes 7 to 9 contain microsomes from rats treated with zolazepam at a dose of 60 mg/kg. Lanes 10 to 12 contain microsomes from rats treated with Telazol® at a dose of 120 mg/kg. Lanes 13 to 18 contain purified rat cytochrome P450 2B1 at a concentration of 1.0, 0.5, 0.5, 0.25, 0.1, and 0.05 pmol per lane, respectively.

Table 3.11. Effects of treatment with Telazol®, tiletamine, and zolazepam on hepatic levels of cytochrome P450 2B1 and cytochrome P450 2B2.

Treatment (Dose)	Cytochrome P450 2B1 content (pmol/mg protein)	Cytochrome P450 2B1 as percentage of total cytochrome P450	Cytochrome P450 2B2 content (pmol/mg protein)	Cytochrome P450 2B2 as percentage of total cytochrome P450
Control	1.3 ± 0.8 (1.0)	0.1 ± 0.0 (1.0)	5.2 ± 1.3 (1.0)	0.4 ± 0.1 (1.0)
Telazol® (120 mg/kg)	94.5 ± 11.7 † (72.7)	7.7 ± 0.9 † (77.0)	47.4 ± 7.6 † (9.1)	3.9 ± 0.6 † (9.8)
Tiletamine (60 mg/kg)	10.3 ± 3.2 (7.9)	0.8 ± 0.2 (8.0)	15.8 ± 1.7 (3.0)	1.2 ± 0.1 (3.0)
Zolazepam (60 mg/kg)	56.5 ± 5.2 † (43.5)	4.6 ± 0.4 † (46.0)	37.6 ± 1.1 † (7.2)	3.0 ± 0.1 † (7.5)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

3.12.3 *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 2E1 IgG*

An immunoblot containing hepatic microsomes from untreated rats and rats treated with Telazol®, tiletamine, or zolazepam was probed with anti-cytochrome P450 2E1 IgG, and is shown in Figure 3.10. Previously prepared pooled liver microsomes from rats induced with ethanol or isoniazid were included on the gel to serve as internal standards, and are represented by the intensely stained bands in lanes 13 and 14, or lanes 15 and 16 of the immunoblot, respectively. There appears to be no difference in staining intensity of the band in lanes (lanes 1 to 12) containing microsomes from untreated and tiletamine-, zolazepam-, and Telazol®-treated rats.

Results of immunoquantitation of cytochrome P450 2E1 in hepatic microsomes prepared from rats treated with Telazol®, tiletamine, or zolazepam are displayed in Table 3.12. A standard curve could not be generated because of a lack of purified cytochrome P450 2E1. Hence, the hepatic level of cytochrome P450 2E1 is presented as integrated intensity per milligram of microsomal protein. The results indicated that there was no significant difference in the amount of immunoreactive cytochrome P450 2E1 protein between control and treatment groups.

3.12.4. *Immunoblot analysis of hepatic microsomes probed with cytochrome P450 3A1 IgG*

An immunoblot of hepatic microsomes from rats treated with Telazol®, tiletamine, or zolazepam was probed with anti-cytochrome P450 3A IgG and is illustrated in Figure 3.11. The antibody reacted with both cytochrome P450 3A1 and cytochrome P450 3A2. Because the two isozymes have the same mass to charge ratio, a single band was detected in each lane and

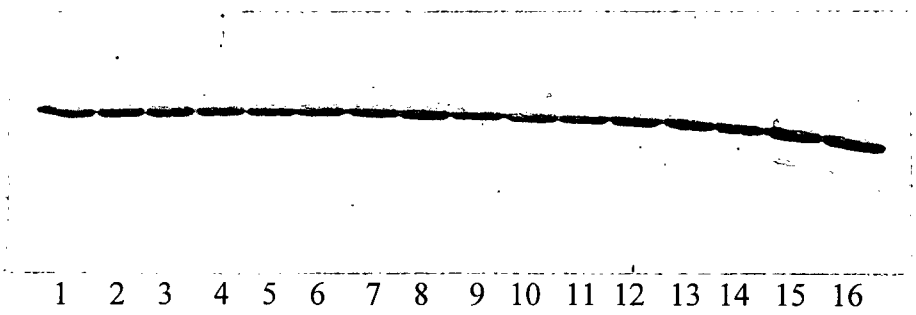

Figure 3.10. Immunoblot of rat hepatic microsomes probed with polyclonal antibody against rat cytochrome P450 2E1. Final concentration of microsomal samples was 10 μg of microsomal protein per lane. Concentration of primary antibody (rabbit anti-cytochrome P450 2E1 IgG) was approximately 10 μg/mL. Dilution of secondary antibody (goat anti-rabbit IgG) was 1:3000. Alkaline phosphatase reaction time was 1.3 min. Lanes 1 to 3 contain microsomes from vehicle-treated rats. Lanes 4 to 6 contain microsomes from rats treated with tiletamine at a dose of 60 mg/kg. Lanes 7 to 9 contain microsomes from rats treated with zolazepam at a dose of 60 mg/kg. Lanes 10 to 12 contain microsomes from rats treated with Telazol® at a dose of 120 mg/kg. Lanes 13 and 14 contain pooled hepatic microsomes from rats treated with ethanol. Lanes 15 and 16 contain pooled hepatic microsomes from rats treated with isoniazid.

Table 3.12. Effects of treatment with Telazol®, tiletamine, and zolazepam on hepatic level of cytochrome P450 2E1.

Treatment (Dose)	Amount of immunoreactive protein
Control	83.4 ± 6.4 (1.0)
Telazol® (120 mg/kg)	67.9 ± 5.1 (0.8)
Tiletamine (60 mg/kg)	71.8 ± 7.8 (0.9)
Zolazepam (60 mg/kg)	79.0 ± 1.7 (0.9)

Each value is the mean ± standard error of the mean for 6 rats per treatment group.
Each value was expressed as the integrated intensity of the band per milligram protein.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
There was no significant difference among the group mean values.

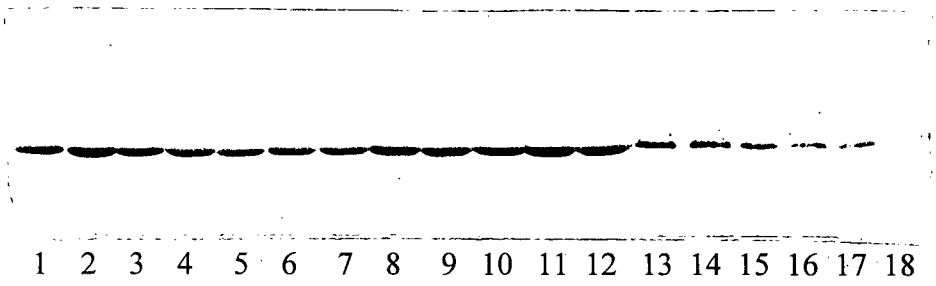

Figure 3.11. Immunoblot of rat hepatic microsomes probed with polyclonal antibody against rat cytochrome P450 3A1. Final concentration of microsomal samples was 10 μg of microsomal protein per lane. Concentration of primary antibody (rabbit anti-cytochrome P450 3A1 IgG) was 50 μg/mL. Dilution of secondary antibody (goat anti-rabbit IgG) was 1:3000. Alkaline phosphatase reaction time was 1.5 min. Lanes 1 to 3 contain microsomes from vehicle-treated rats. Lanes 4 to 6 contain microsomes from rats treated with tiletamine at a dose of 60 mg/kg. Lanes 7 to 9 contain microsomes from rats treated with zolazepam at a dose of 60 mg/kg. Lanes 10 to 12 contain microsomes from rats treated with Telazol® at a dose of 120 mg/kg. Lanes 13 to 18 contain purified rat cytochrome P450 3A1 at a concentration of 0.5, 0.375, 0.25, 0.125, 0.0625, and 0.0312 pmol per lane, respectively.

Table 3.13. Effects of treatment with Telazol®, tiletamine, and zolazepam on hepatic levels of cytochrome P450 3A isozymes.

Treatment (Dose)	Cytochrome P450 3A content (pmol/mg protein)	Cytochrome P450 3A as percentage of total cytochrome P450
Control	40.0 ± 4.4 (1.0)	2.9 ± 0.2 (1.0)
Telazol® (120 mg/kg)	92.1 ± 13.3 † (2.3)	7.5 ± 1.0 † (2.6)
Tiletamine (60 mg/kg)	35.4 ± 3.0 (0.9)	2.7 ± 0.2 (0.9)
Zolazepam (60 mg/kg)	72.0 ± 8.0 † (1.8)	5.9 ± 0.8 † (2.0)

Each value is the mean ± standard error of the mean for 6 rats per treatment group and included both cytochromes P450 3A1 and P450 3A2.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

represented the sum of both cytochromes P450 3A1 and P450 3A2. Various amounts of purified cytochrome P450 3A1 were included on the gel for construction of a standard curve (lanes 13 to 18). The bands in lanes containing hepatic microsomes from rats treated with zolazepam and Telazol® appeared to be slightly darker than those of hepatic microsomes from untreated rats and rats treated with tiletamine.

Mean hepatic levels of cytochrome P450 3A (cytochrome P450 3A1 plus cytochrome P450 3A2) for the control and treatment groups are shown in Table 3.13. Telazol® caused a greater than two-fold increase in the hepatic level of cytochrome P450 3A, while zolazepam produced a two-fold increase. In contrast, treatment with tiletamine had no significant effect on cytochrome P450 3A protein levels.

3.13. *RESULTS OF DETERMINATION OF METABOLITE COMPLEX FORMATION*

Results from sections 1 and 2 indicated that the extent of increase in hepatic levels of cytochrome P450 2B isozymes produced by Telazol® was consistently greater than that observed in cytochrome P450 2B-mediated enzyme activities. The inconsistency could be due to binding of drug metabolite to cytochrome P450, thereby inactivating the enzyme and consequently leading to a decrease in enzyme activites. In order to investigate the possibility of metabolite complex formation, total cytochrome P450 content was determined for hepatic microsomes from a control rat that was preincubated with Telazol® and NADPH. The resulting cytochrome P450 content (not shown) was similar to that determined in the absence of NADPH with or without Telazol®. In addition, total cytochrome P450 content was also determined for hepatic microsomes from a rat treated with Telazol® at a dose of 120 mg/kg, in

the presence or absence of potassium ferricyanide, which is known to dissociate cytochrome P450-metabolite complexes. The results (not shown) indicated that the reducing agent had no effect on total cytochrome P450 content.

SECTION 3:

Results of previous sections indicated that Telazol® and its constituents had an inductive effect on cytochrome P450 similar to that of PB, a protypical cytochrome P450 2B inducer. In this section, a single dose of PB of 60 or 120 mg/kg was administered to rats in order to mimic the dose used for Telazol®, tiletamine, or zolazepam. The inductive effect produced by PB 24 h after drug exposure was compared with that of the three agents at a similar dose and dosing regimen.

3.14. *EFFECTS OF A SINGLE TREATMENT WITH PHENOBARBITAL ON BODY WEIGHT AND LIVER WEIGHT IN RATS*

The effects of a single i.p. treatment with PB on body and liver weight in rats are presented in Table 3.14. A significant increase in liver weight and liver weight expressed as a percentage of body weight was found for rats treated with phenobarbital at doses of 60 and 120 mg/kg, compared to the control group.

3.15. *EFFECTS OF A SINGLE TREATMENT WITH PHENOBARBITAL ON PROTEIN CONCENTRATION AND TOTAL HEPATIC CYTOCHROME P450 CONTENT*

The data shown in Table 3.15 indicated that a single treatment with PB at doses of 60 and 120 mg/kg increased microsomal protein concentration, cytochrome P450 concentration and total cytochrome P450 content significantly. There was no difference in the magnitude of effects produced by the two doses.

Table 3.14. Effects of a single treatment with phenobarbital on body weight and liver weight in rats.

Dose of phenobarbital (mg/kg)	Body weight (g)	Liver weight (g)	Liver weight as percentage of body weight
Control	285 ± 9	13.6 ± 0.5	4.8 ± 0.1
60	294 ± 4	14.7 ± 0.3	5.0 ± 0.1 †
120	304 ± 3	16.3 ± 0.3 †	5.4 ± 0.1 †

Each value is the mean ± standard error of the mean for 5 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

Table 3.15. Effects of a single treatment with phenobarbital on protein concentration and total hepatic cytochrome P450 content.

Dose of phenobarbital (mg/kg)	Protein concentration (mg/mL)	Total cytochrome P450 concentration (nmol/mL)	Total cytochrome P450 content (nmol/mg protein)
Control	20.1 ± 0.6 (1.0)	25.8 ± 1.4 (1.0)	1.3 ± 0.1 (1.0)
60	22.8 ± 0.7 † (1.1)	40.9 ± 2.5 † (1.6)	1.8 ± 0.1 † (1.4)
120	23.0 ± 0.6 † (1.1)	44.0 ± 2.0 † (1.7)	1.9 ± 0.1 † (1.5)

Each value is the mean ± standard error of the mean for 5 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

3.16. *EFFECTS OF A SINGLE TREATMENT WITH PHENOBARBITAL ON BROD, EROD, AND PROD ACTIVITIES*

BROD, EROD, PROD activities of hepatic microsomes prepared from rats treated with a single dose of PB are shown in Table 3.16. Treatment with PB at a dose of 60 mg/kg and 120 mg/kg increased BROD activity by 22- and 23-fold, respectively. The same treatment caused a two-fold increase in EROD activity, and six- and seven-fold increases, respectively, in PROD activity. The extent of increase in the enzyme activities was similar between the two treatment groups.

3.17. *EFFECTS OF A SINGLE TREATMENT WITH PHENOBARBITAL ON TESTOSTERONE HYDROXYLASE ACTIVITIES*

Mean testosterone hydroxylase activities of hepatic microsomes prepared from control rats and rats treated with a single dose of PB at 60 or 120 mg/kg are indicated in Table 3.17. There was a significant decrease in testosterone 2α-hydroxylation in hepatic microsomes from rats treated with phenobarbital at a dose of 120 mg/kg, while the rate of testosterone 7α-hydroxylation remained unchanged. Increases of 1.6- and 1.7-fold for testosterone 6β-hydroxylase activity and increases of 13- and 17-fold for testosterone 16β-hydroxylase activity were observed with hepatic microsomes from rats treated with PB at a dose of 60 mg/kg and 120 mg/kg, respectively. Drug treatment had no significant effect on testosterone 16α-hydroxylation. Both treatment groups produced a two-fold increase in the rate of androstenedione production.

Table 3.16. Effects of a single treatment with phenobarbital on BROD, EROD, and PROD activities.

Dose of phenobarbital (mg/kg)	BROD activity (pmol/mg/min)	EROD activity (pmol/mg/min)	PROD activity (pmol/mg/min)
Control	323 ± 22 (1.0)	529 ± 50 (1.0)	380 ± 45 (1.0)
60	6974 ± 118 † (21.6)	1035 ± 55 † (2.0)	2347 ± 110 † (6.2)
120	7292 ± 56 † (22.6)	1037 ± 92 † (2.0)	2657 ± 132 † (7.0)

Each value is the mean ± standard error of the mean for 5 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

Table 3.17. Effects of a single treatment with phenobarbital on testosterone hydroxylase activities.

Dose of phenobarbital (mg/kg)	Testosterone metabolites (pmol metabolite formed/min/mg protein)					
	2α	6β	7α	16α	16β	androstenedione
Control	2017 ± 161 (1.0)	2246 ± 278 (1.0)	261 ± 35 (1.0)	2820 ± 237 (1.0)	179 ± 8 (1.0)	1547 ± 63 (1.0)
60	1743 ± 195 (0.9)	3512 ± 233 † (1.6)	260 ± 28 (1.0)	2829 ± 203 (1.0)	2398 ± 125 † (13.4)	2604 ± 318 † (1.7)
120	1154 ± 113 † (0.6)	3748 ± 433 † (1.7)	339 ± 23 (1.3)	3161 ± 125 (1.1)	3123 ± 52 † (17.4)	2673 ± 49 † (1.7)

Each value is the mean ± standard error of the mean for 5 rats per treatment group.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

3.18. *IMMUNOBLOT ANALYSIS OF HEPATIC MICROSOMES PREPARED FROM RATS TREATED WITH A SINGLE DOSE OF PHENOBARBITAL*

Information on the effect of a single dose of PB on hepatic levels of various cytochromes P450 is very limited at the present time. In this study, the effects of a single treatment of PB on hepatic contents of cytochrome P450 2B and P450 3A isozymes were determined, and the results are shown below.

3.18.1. *Immunoblot analysis of hepatic microsomes probed with anti-cytochrome P450 2B1 IgG*

Immunoblots of hepatic microsomes from rats treated wth a single dose of PB were prepared and quantitated. Because of the dramatic increase in the size and staining intensity of immunoreactive bands corresponding to cytochrome P450 2B1 in lanes that contained microsomal samples from PB-treated rats, it was very difficult to differentiate between the cytochrome P450 2B1 and cytochrome P450 2B2 bands (Figure 3.12). Hence, the combined content of the two isozymes is reported. Results of immunoquantitation indicated a significant increase of approximately 25-fold in hepatic microsomes from rats treated with PB at a dose of 60 or 120 mg/kg (Table 3.18), hence, the two doses examined had a similar effect on the hepatic contents of cytochrome P450 2B isozyme.

3.18.2. *Immunoblot analysis of hepatic microsomes probed with cytochrome P450 3A1 IgG*

An immunoblot of hepatic microsomes from rats treated with a single dose of PB is illustrated in Figure 3.13. An apparent increase was found in the staining intensity of the bands in lanes containing hepatic microsomes from rats treated with PB, and the increase appeared to be similar between the two doses studied.

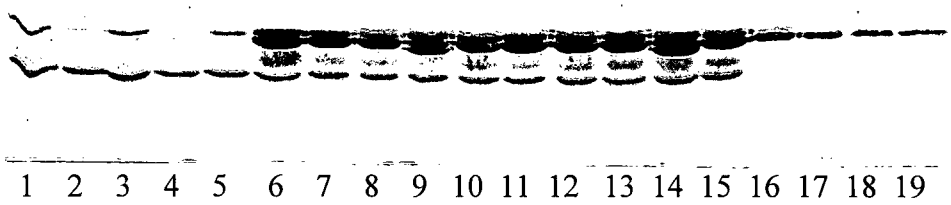

Figure 3.12. Immunoblot of rat hepatic microsomes probed with polyclonal antibody against rat cytochrome P450 2B1. Final concentration of microsomal samples was 10 μg of microsomal protein per lane. Concentration of primary antibody (rabbit anti-cytochrome P450 2B1 IgG) was 2 μg/mL. Dilution of secondary antibody (goat anti-rabbit IgG) was 1:3000. Alkaline phosphatase reaction time was 0.9 min. Lanes 1 to 5 contain microsomes from vehicle-treated rats. Lanes 6 to 15 contain microsomes from rats treated with phenobarbital at a dose of 60 mg/kg (lanes 6 to 10), or 120 mg/kg (lanes 11 to 15). Lanes 16 to 19 contain purified rat cytochrome P450 2B1 at a concentration of 1.0, 0.5, 0.25, and 0.1 pmol per lane, respectively.

Table 3.18. Effects of a single treatment with phenobarbital on hepatic levels of cytochrome P450 2B isozymes.

Dose of phenobarbital (mg/kg)	Cytochrome P450 2B content (pmol/mg protein)	Cytochrome P450 2B as percentage of total cytochrome P450
Control	7.9 ± 2.3 (1.0)	0.6 ± 0.2 (1.0)
60	195.7 ± 10.7 † (24.8)	11.0 ± 0.7 † (18.3)
120	196.9 ± 23.7 † (24.9)	10.2 ± 0.9 † (17.0)

Each value is the mean ± standard error of the mean for 5 rats per treatment group and included both cytochromes P450 2B1 and P450 2B2.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

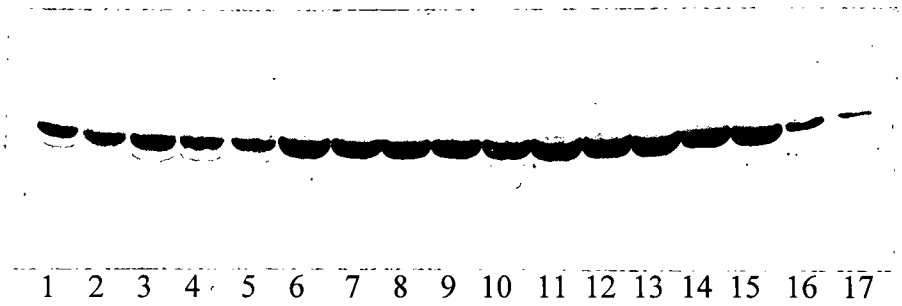

Figure 3.13. Immunoblot of rat hepatic microsomes probed with polyclonal antibody against rat cytochrome P450 3A1. Final concentration of microsomal samples was 10 μg of microsomal protein per lane. Concentration of primary antibody (rabbit anti-cytochrome P450 3A1 IgG) was 50 μg/mL. Dilution of secondary antibody (goat anti-rabbit IgG) was 1:3000. Alkaline phosphatase reaction time was 3.5 min. Lanes 1 to 5 contain microsomes from vehicle-treated rats. Lanes 6 to 15 contain microsomes from rats treated with phenobarbital at a dose of 60 mg/kg (lanes 6 to 10), or 120 mg/kg (lanes 11 to 15). Lanes 16 and 17 contain purified rat cytochrome P450 3A1 at a concentration of 0.5 and 0.25 pmol per lane, respectively.

Table 3.19. Effects of a single treatment with phenobarbital on hepatic levels of cytochrome P450 3A isozymes.

Dose of phenobarbital (mg/kg)	Cytochrome P450 3A content (pmol/mg protein)	Cytochrome P450 3A as percentage of total cytochrome P450
Control	18.1 ± 2.2 (1.0)	1.4 ± 0.1 (1.0)
60	137.0 ± 1.2 † (7.6)	7.8 ± 0.5 † (5.5)
120	162.0 ± 9.1 † (8.9)	8.5 ± 0.3 † (6.0)

Each value is the mean ± standard error of the mean for 5 rats per treatment group and included both cytochromes P450 3A1 and P450 3A2.
Control group was treated with vehicle at a dose of 1 mL/kg.
Values in parenthesis indicate the relative difference to the control group.
† Mean value of the treatment group was significantly different ($p < 0.05$) from that of the control group.

Combined hepatic levels of cytochromes P450 3A1 and P450 3A2 are indicated in Table 3.19. A single dose of PB at 60 and 120 mg/kg increased the hepatic level of cytochrome P450 3A isozymes by eight- and nine-fold, respectively.

4. DISCUSSION

Telazol® has been used widely as an anesthetic and immobilizing agent in veterinary medicine for over a decade, but there is no information available regarding the effect of the drug, or its constituents, on hepatic cytochromes P450. The purpose of the present study was to examine the effects of treatment with Telazol®, tiletamine, and zolazepam on hepatic cytochrome P450 expression in rats, and to compare the effects produced with those of PB, when administered using the same dosing regiment. Effects of treatment with these agents are discussed according to their impact on liver and body weight, total cytochrome P450 content, various enzyme activities, and hepatic contents of specific cytochrome P450 isozymes.

4.1. *EFFECTS OF TREATMENT ON BODY WEIGHT, LIVER WEIGHT, AND TOTAL CYTOCHROME P450 CONTENT IN RATS*

Unlike PB treatment, administration of Telazol®, tiletamine, or zolazepam had no effect on rat liver weight or body weight. Treatment with PB after a single dose of 60 or 120 mg/kg significantly increased liver weight as a percentage of body weight. It is well documented that liver enlargement and proliferation of smooth endoplasmic reticulum accompanies repeated PB treatment (37). For instance, treatment with PB at a dose of 25 mg/kg or greater for 6 days elevated the liver to body weight ratio significantly by 1.1-fold in rats (142), while a 1.3-fold increase was seen in animals treated with PB at a dose of 500 ppm in the diet for 14 days (143). Treatment with benzodiazepines at high doses has also been shown to cause liver hypertropy in mice and rats. An significant increase of 1.1-fold in liver to body weight ratio was found in mice treated with dietary oxazepam at a dose of 125 ppm and greater for 7 days (131), while the

same fold increase in liver weight as a percentage of body weight was seen in rats treated with chlordiazepoxide at a dose of 100 mg/kg for 5 days (128).

Elevation of hepatic cytochrome P450 content is commonly observed in microsomes prepared from animals treated with PB. Treatment with PB at a dose of 100 mg/kg for 3 days has been shown to produce a significant increase in microsomal cytochrome P450 content (144), while a single treatment at the same dose increased the level of cytochrome P450 significantly within 18 h after drug exposure (145). Previous studies have shown that treatment with benzodiazepines also caused an increase in microsomal cytochrome P450 content. For instance, dietary treatment with oxazepam at a dose of 2500 ppm for 7 days increased total cytochrome P450 content significantly by 1.6-fold in mice (131). The results from the present study indicated that neither Telazol®, tiletamine, or zolazepam at doses tested, had any effect on total hepatic cytochrome P450 content. However, a single i.p. treatment with PB at a dose of 60 or 120 mg/kg produced a significant increase in hepatic cytochrome P450 content, indicating induction of hepatic cytochromes P450.

4.2. *EFFECTS OF TREATMENT ON CYTOCHROME P450-MEDIATED MONOOXYGENASE ACTIVITIES*

The cytochrome P450 isozymes responsible for catalyzing dealkylation of the homologous series of alkoxyresorufins, which includes benzyloxy, ethoxy and pentoxyresorufin, are highly specific for the type of inducers present. With untreated rat liver microsomes, for instance, BROD activity is catalyzed mainly by cytochromes P450 2B1 and P450 2C11, and to a lesser extent, by cytochromes P450 1A2, P450 2C6, and P450 3A (146). In hepatic microsomes from PB-treated rats, however, the majority of the activity is contributed

by cytochrome P450 2B1 (146). Similarly, no single form of cytochrome P450 dominates in catalyzing PROD activity in hepatic microsomes of untreated rats. Cytochromes P450 2C6, P450 2C11, and P450 3A all play a role in mediating PROD activity (146). Cytochrome P450 2B1 is the major catalyst of the reaction in rats treated with PB (146). Treatment with PB has been found to increase BROD and PROD activities by more than 100- and 200-fold, respectively (146).

Results of the present study show that Telazol® produced dose-dependent increases in both BROD and PROD activities suggesting that expression of cytochrome P450 2B isozymes was induced. Treatment with Telazol® at a dose of 120 mg/kg elicited a larger increase in BROD activity in section 2 as compared to section 1. The lower enzyme activity of control samples from the former section might account for the difference. The results of section 2 also suggest that zolazepam was the main constituent responsible for the enzyme induction observed with Telazol® treatment, although the inductive effect of zolazepam was less than that produced by Telazol®. Treatment with tiletamine resulted in a small (approximately two-fold) increase in BROD activity. However, the sum of the activities of the tiletamine- and zolazepam-treated groups was less than that of the Telazol®-treated group. Statistical analysis indicated that the BROD activity of hepatic microsomes from rats treated with tiletamine or zolazepam was significantly different from that of Telazol®-treated group, implying the combination of the two constituents, Telazol®, exerted a synergistic inductive effect on cytochrome P450 expression. Further testing with two way Analysis of Variance indicated no significant interaction between tiletamine and zolazepam, although the power of the statistical test was very low, suggesting a high probability of commiting a Type II error.

A single dose of PB, at 60 or 120 mg/kg, produced a significant increase in both BROD and PROD activities. However, the magnitude of increase in PROD activity was much smaller than the fold increase in BROD activity. In fact, this trend was seen in the previous section also. The PROD activity of hepatic microsomes from untreated rats in all three sections appeared to be very high compared to literature values, which were 15- to 30-fold smaller than the values obtained in this study (146). The relatively high level of PROD activity of the untreated groups could have masked the inductive effect produced by drug treatment, but the reason(s) for the high basal PROD activity are presently unknown. Both BROD and PROD activities were measured under optimal assay conditions where rate of product formation was linear with respect to time and microsomal protein concentration.

In the present study, BROD activity was a superior indicator of cytochrome P450 2B induction. Overall, the results of the BROD and PROD assays demonstrated that a single dose of PB at 60 or 120 mg/kg elicited a greater inductive effect on cytochrome P450 2B than treatment with Telazol® and its constituents using the same dosing regimen, although the drugs were administered in separate treatment sets. In addition, the induction produced by PB was very similar between the two doses examined.

EROD activity is considered to be a selective indicator of cytochrome P450 1A induction, the reason being that a significant 50- to 60-fold increase is observed typically in hepatic microsomes from rats treated with cytochrome P450 1A inducers such as 3-MC (146). Treatment with PB could also produce a small but significant increase of three to six-fold in EROD activity (146). With untreated rat liver microsomes, EROD is catalyzed mainly by cytochrome P450 2C6, and by a combination of cytochromes P450 1A1 (147), P450 2C11, and

P450 3A (146). The majority of EROD activity is mediated by cytochrome P450 1A1 in hepatic microsomes of animals treated with 3-MC, and by cytochrome P450 2B1 in rats treated with PB (146). The small but significant increase (1.5-fold) in EROD activity of hepatic microsomes from rats treated with the highest dose of Telazol® in section 1 might indicate induction of cytochrome P450 2B isozymes. Similarly, treatment with PB at a single dose of 60 or 120 mg/kg resulted in two-fold increase in EROD activity. In contrast, results from experimental section 2 suggested a lack of effect produced by Telazol®, tiletamine, or zolazepam on EROD activity.

p-Nitrophenol hydroxylation is commonly used as an indicator of cytochrome P450 2E1 activity. Induction of cytochrome P450 2E1 by compounds such as acetone has been shown to correlate with increased in *p*-nitrophenol hydroxylase activity in various species including rabbit, mouse, hamster, and rat (148). In this study, treatment with increasing doses of Telazol® did not significantly increase *p*-nitrophenol hydroxylase activity, indicating that the drug likely had no effect on hepatic expression of cytochrome P450 2E1.

It has been well documented that various forms of hepatic cytochrome P450 catalyze the oxidation of testosterone with a high degree of regio- and stereoselectivity (53). As a result, testosterone hydroxylation has been used as a marker assay for various cytochrome P450 isozymes. The metabolites of interest in this study include 2α-, 6β-, 7α-, 16α- and 16β-hydroxytestosterone, and androstenedione. Each metabolite was resolved by a HPLC system and identified according to its retention time. The rate of metabolite formation was determined and served as an indicator of the level of the cytochrome(s) P450 responsible for the specific oxidative pathway. For instance, testosterone 2α-hydroxylation has been used as a probe for

the activity of cytochrome P450 2C11, a male-specific isozyme, which is also involved in testosterone 16α-hydroxylation and testosterone 17-oxidation to produce androstenedione (53). Cytochrome P450 3A and cytochrome P450 2A1 are mainly responsible for testosterone 6β- and 7α-hydroxylation, respectively, while cytochrome P450 2B catalyzes testosterone 16α- and 16β-hydroxylation, and testosterone 17-oxidation (53).

The pattern of testosterone metabolites produced by microsomes from rats treated with Telazol® was similar to that elicited by microsomes from PB-treated rats. The greatest effect of treatment with Telazol® was seen with testosterone 16β-hydroxylase activity, which is catalyzed by cytochromes P450 2B1 and P450 2B2. Results from section 2 demonstrated that zolazepam, but not tiletamine, also induced this enzyme activity. Testosterone 16β-hydroxylase activity appeared to be greater after Telazol® treatment at the maximal dose tested than the sum of the activities after tiletamine and zolazepam treatment. This may indicate an additive effect of tiletamine and zolazepam, when combined in Telazol®, in triggering cytochrome P450 induction. However, results of two way ANOVA displayed no significant interaction between the two constituents, although the sample size may be too small to detect a significant difference. PB at a single dose of 60 and 120 mg/kg induced testosterone 16β-hydroxylase activity by approximately 13- and 17-fold, respectively. In comparison, treatment with Telazol®, at a dose of 120 mg/kg, induced testosterone 16β-hydroxylase activity by approximately eight-fold.

Treatment with the tiletamine, zolazepam, or Telazol® at a dose of 120 mg/kg significantly decreased testosterone 2α-hydroxylase activity, indicating a suppression of cytochrome P450 2C11. Previous studies have shown that expression of cytochrome P450

2C11 is often suppressed following treatment with compounds that are strong inducers of cytochrome P450 1A, P450 2B, or P450 3A (149). Treatment of mature male rats with 3-MC or PB, for instance, resulted in decreased testosterone 2α-hydroxylase activity to a level that was approximately half that of untreated male rats (53). The mechanism of enzyme suppression is presently unknown. In the present study, PB treatment at a single dose of 120 mg/kg resulted in a significant decrease in testosterone 2α-hydroxylase activity but not in testosterone 16α-hydroxylase activity. Testosterone 16α-hydroxylation is catalyzed by both cytochrome P450 2B and cytochrome P450 2C11 (53), hence, this activity reflects the contribution of both isozymes. The decrease in testosterone 16α-hydroxylase activity produced by Telazol®, tiletamine, and zolazepam might be a result of cytochrome P450 2C11 suppression combined with induction of cytochrome P450 2B isozymes. Suppression of cytochrome P450 2C11 was likely greater than the induced expression of cytochrome P450 2B. Treatment with PB had no significant effect on testosterone 16α-hydroxylase activity in the present study, although a small increase has been reported in the literature after repeated treatment (53), suggesting that the induction of cytochrome P450 2B offset the suppression of cytochrome P450 2C11. Purified cytochromes P450 2B1 and P450 2B2, as well as cytochrome P450 2C11, have also been shown to be effective catalyst of androstenedione formation (43). No effect on the rate of androstenedione formation was found in hepatic microsomes from rats treated with Telazol®, tiletamine, or zolazepam, while a two-fold increase was seen in hepatic microsomes from rats treated with PB. The lack of change in the rate of androstenedione formation following administration of Telazol®, tiletamine, and zolazepam again likely reflects

suppression of cytochrome P450 2C11 combined with induction of cytochromes P450 2B1 and P450 2B2.

No significant change in testosterone 7α- or 6β-hydroxylase activities was found in hepatic microsomes from rats treated with Telazol®, tiletamine, or zolazepam. Treatment with PB at a single dose of 60 or 120 mg/kg elevated testosterone 6β-hydroxylase activity significantly, indicating induction of cytochrome P450 3A, while no significant effect was observed with testosterone 7α-hydroxylase activity, suggesting unaltered cytochrome P450 2A1 expression. Earlier studies reported that multiple treatment with PB elicited an increase of approximately two-fold in testosterone 6β- and 7α-hydroxylase activities (53).

4.3. *EFFECTS OF TREATMENT ON HEPATIC LEVELS OF SPECIFIC CYTOCHROMES P450*

The enzyme activity data indicated that treatment with Telazol® increased cytochrome P450 2B-mediated monooxygenase activities but not cytochrome P450 1A-, P450 2E-, or P450 3A-mediated activities. To determine if Telazol® was inducing expression of the various cytochromes at the protein level, immunoblot analysis was conducted.

4.3.1. *Cytochrome P450 1A*

Immunoblots probed with anti-cytochrome P450 1A1 IgG indicated that treatment with Telazol®, tiletamine, or zolazepam had no effect on the expression of cytochrome P450 1A1 or P450 1A2. The results suggested that the observed increase in EROD activity in section 1 was likely an effect produced by induction of cytochrome P450 2B isozymes.

4.3.2. *Cytochrome P450 2B*

Densitometric quantitation of immunoblots probed with anti-cytochrome P450 2B1 IgG demonstrated that Telazol® induced expression of cytochrome P450 2B isozymes. However, the extent of increase observed with microsomes from section 2 was different from that obtained with microsomes in section 1. While there was a 17-fold increase in the hepatic level of cytochrome P450 2B1 for rats treated with Telazol® at a dose of 120 mg/kg in the first treatment set, a 73-fold increase was produced by the same treatment in section 2. The discrepancy between the two treatment sets cannot be explained. In both sections, the fold increase in cytochrome P450 2B1 levels was greater than that of enzyme activities for the same treatment groups. There was no evidence of enzyme-metabolite complex formation or increased levels of inactive cytochrome P450 (i.e. P420) in microsomes prepared from Telazol®-treated rats. Cytochrome P450 2B may be involved in the metabolism of either tiletamine or zolazepam, which in turn masked the full induction of cytochrome P450 2B-mediated enzyme activities. Previous studies had shown an increase in the metabolism of ketamine after phenobarbital pretreatment in rats and rabbits, indicating phenobarbital-inducible forms of cytochrome P450 may be involved in the metabolism of this drug (150). In

addition, it had been reported that cytochrome P450 2B isozymes may play a role in the metabolism of diazepam in rat hepatic microsomes (151). Immunoblot analysis also indicated that treatment with zolazepam induced hepatic expression of cytochrome P450 2B1 protein, but to a smaller extent than Telazol® treatment. Statistical analysis of the data indicated that the level of cytochrome P450 2B1 in hepatic microsomes prepared from rats treated with tiletamine or zolazepam was significantly different from that of Telazol®-treated group. As found with the enzyme activities, results of two way ANOVA showed no significant interaction between tiletamine and zolazepam. A larger sample size would be necessary to draw a more reliable conclusion from the statistical analysis.

The hepatic level of cytochrome P450 2B2 was increased for rats treated with Telazol® at a dose of 120 mg/kg, although the extent of increase noted in section 1 was not statistically significant. Zolazepam, but not tiletamine, also had an inductive effect on the hepatic level of cytochrome P450 2B2 that was almost as great as that produced by Telazol®. Studies have shown that induction of cytochrome P450 2B1 is accompanied by increased expression of cytochrome P450 2B2 (152, 153, 154). Certain inducers may alter the proportion of cytochrome P450 2B1 to P450 2B2, but selective induction of one of these isozymes without co-induction of the other form has not been reported (147). Hence, treatment with both Telazol® and zolazepam appeared to induce the expression of cytochrome P450 2B2.

A single dose of PB at 60 or 120 mg/kg dramatically increased the hepatic level of cytochrome P450 2B isozymes. It is interesting to note that the inductive effects produced by each of the two doses were similar with respect to the results of both enzyme assays and immunoquantitation. However, the inductive effect produced by a single treatment with PB

was smaller than that reported by Yamazoe *et al.* (155). In that study, a greater than 200-fold increase in the protein levels of cytochrome P450 2B isozymes was seen in hepatic microsomes from rats treated with PB at a dose of 80 mg/kg for 3 consecutive days (155).

4.3.3. *Cytochrome P450 2E*

Treatment with Telazol®, tiletamine, or zolazepam had no effect on the hepatic level of cytochrome P450 2E1. Results of immunoblot analysis support the enzyme activity data that treatment with Telazol® did not alter the rate of *p*-nitrocatechol formation. This is consistent with the literature where PB or PB-like inducers did not display any inductive effect on cytochrome P450 2E1 expression.

4.3.4. *Cytochrome P450 3A*

Althought neither treatment with Telazol® or zolazepam significantly altered testosterone 6β-hydroxylase activity, immunoblot analysis indicated that both compounds induced expression of cytochrome P450 3A isozymes. Hepatic levels of cytochrome P450 3A isozymes have been shown to be induced by PB treatment. PB at a dose of 75 mg/kg for 3 to 4 days was reported to induce cytochrome P450 3A1 and cytochrome P450 3A2 contents by greater than 10-fold and 2-fold, respectively (72). In another study, a 45-fold increase in the hepatic level of cytochrome P450 3A isozymes was seen with PB treatment at a dose of 75 mg/kg for 4 days (156). In the present study, a single dose of PB of 60 or 120 mg/kg significantly induced hepatic level of cytochrome P450 3A by eight- and nine-fold,

respectively. Hence, the two doses produced a similar inductive effect on cytochrome P450 3A expression.

4.3.5. *Comparison of the effects of treatment on hepatic contents of specific cytochrome P450 isozymes*

Results of immunoblot analysis indicated that treatment with Telazol® and zolazepam affected specific cytochrome P450 isozymes in a manner similar to that of PB. Treatment with all three agents significantly induced the hepatic expression of cytochrome P450 2B, and slightly induced the hepatic expression of cytochrome P450 3A. Likewise, treatment with PB, Telazol®, or its constituents had no effect on cytochrome P450 1A, or P450 2E expression. Hence, Telazol® and zolazepam can be classified as "PB-type" inducers, although in all cases, their inductive effects were weaker than those produced by PB.

4.4. *EFFECTS OF TREATMENT WITH TELAZOL® ON ANTIBODY INHIBITION OF BROD AND PROD ACTIVITIES*

Previous studies indicated that cytochrome P450 2B isozymes are major mediators of BROD activity in hepatic microsomes of untreated rats and that they contribute up to 70% of the enzyme activity (146). On the other hand, cytochrome P450 2B isozymes are responsible for less than 20% of PROD activity in hepatic microsomes of untreated rats (146) because of their low hepatic content, which has been reported to be less than 4% of total cytochrome P450 (152). The results of antibody inhibition of BROD activity are in close agreement with the literature in that maximal inhibition of enzyme activity by antibody to cytochrome P450 2B1 was approximately 70% in microsomes from untreated rats. However, PROD activity was

inhibited by the antibody to a much greater extent than that found previously. As shown in Figure 3.8, the decrease in PROD activity did not level off in the presence of high concentration of anti-cytochrome P450 2B1 IgG, but decreased progressively with increasing concentrations of antibody. Because of the antibody, which was not back-absorbed, could crossreact with cytochrome P450 2C isozymes at high concentration, the continuous decline in the enzyme activity might have been a result of inhibition of cytochrome P450 2C6, which contributes 20% to 40% of PROD activity in hepatic microsomes of untreated rats (146).

BROD and PROD activities have been shown to be catalyzed exclusively by cytochromes P450 2B1 and P450 2B2 in hepatic microsomes of rats treated with PB (146). The results of the present study indicated that in hepatic microsomes from rats treated with Telazol® at a dose of 120 mg/kg, cytochrome P450 2B isozymes contributed more than 90% of both BROD and PROD activities. The increased contribution of cytochrome P450 2B isozymes to both activities correlates with the increase in cytochrome P450 2B1 and P450 2B2 content in hepatic microsomes of Telazol®-treated rats as determined by immunoquantitation.

4.5. *INTERSPECIES COMPARISON OF THE EFFECTS OF TELAZOL® ON HEPATIC CYTOCHROMES P450*

The primary goal of this research project was to determine the effect of Telazol® on hepatic cytochromes P450 in rats. The results can be used to predict, in a crude fashion, the effect of Telazol® on hepatic cytochrome P450 expression in polar bears. The results indicated that Telazol®, at a dose of 80 mg/kg or greater, induced the expression of cytochrome P450 2B isozymes in rats. The doses at which induction occurred were very high compared with those used to produce anesthesia in rats. Previous studies indicated that a dose that ranging from 20 to 40 mg/kg was sufficient to achieve satisfactory analgesia and anesthesia in rats (157, 158). In general, larger species tend to require a lesser dose on a per weight basis than smaller species because of their slower metabolic rate (120). For instance, optimal immobilization was produced in polar bears at a dose of 5 to 8 mg/kg (115, 119). As an approximation, the pharmacological effects of Telazol® in rats can be correlated with that in polar bears by comparing the onset and duration of drug action. In rats, a dose of 40 mg/kg resulted in an mean onset of anesthesia of 2.2 min with an average duraton of 47.7 min (157). In polar bears, an average dose of 5.1 mg/kg had a mean onset of immobilization of 5 min with a duration of approximately 2 h (119). In another study, Telazol® treatment at a dose of 7.9 mg/kg produced an onset of immobilization of 5.3 min and a mean duration of 54.5 min (115). Therefore, a dose equivalent to 80 mg/kg of Telazol® would be too high for use in polar bears. In an earlier study in which hepatic cytochrome P450 expression in polar bears was proposed as a biomarker of arctic contamination (2, 3), the animals were immobilized with Telazol® at a mean dose of 8.9 mg/kg. Because polar bears are physically much larger than rats, have a different metabolic rate, and the scaling factor for cytochrome P450 induction in polar bears based on rat data is

presently unknown, the dose of Telazol® required to produce a similar inductive effect in polar bears cannot be extrapolated from the results of the present study. It should be noted that pharmacological effect of a drug may not be linearly correlated with its effect on hepatic cytochromes P450. Furthermore, the isozymes involved in drug metabolism may not be the same for both species. Nevertheless, the results of this research project can be used as a reference point for studying the effect of Telazol® on cytochrome P450 expression in other animal species.

4.6. *CONCLUSIONS*

This is the first report of the effects of Telazol® treatment on hepatic cytochromes P450. Results of this research study indicated that Telazol® induced the expression of cytochrome P450 2B isozymes in rats in a dose-dependent manner. The pattern of induction elicited by Telazol® was similar to that produced by PB in that at high doses, it had little or no inductive effect on cytochrome P450 1A or cytochrome P450 2E expression, and it induced the expression of cytochrome P450 3A isozymes slightly. However, comparison of results obtained with a single treatment with PB suggested that Telazol®, at a similar doses, exerted a weaker inductive effect on cytochromes P450 as compared with PB. A study on the effect of individual constituents of Telazol® on cytochromes P450 indicated that zolazepam was the main constituent responsible for enzyme induction, while a small inductive effect that was often not statistically significant, resulted from treatment with tiletamine. The results also imply that tiletamine and zolazepam might have acted synergistically when in combined form, although the effect of interaction between the two agents has not shown to be statistically significant. Finally, treatment with PB at a dose of 60 mg/kg or 120 mg/kg elicited an equivalent inductive effect on hepatic cytochrome P450 expression, and a single injection of PB at these doses was effective in producing a 20- to 25-fold increase in cytochrome P450 2B-mediated enzyme activity and protein levels.

4.7. *FUTURE STUDIES*

There are two primary objectives that have yet to be achieved in this study:

1. the effect of interaction of tiletamine and zolazepam on hepatic cytochromes P450;
2. the time-course of cytochrome P450 induction produced by Telazol® in a typical immobilization procedure.

The results of this study implied that the use of tiletamine and zolazepam in combination might have an additive inductive effect on cytochrome P450 expression. Because of the small sample size, statistical analysis was not able to detect a significant difference in the interaction of the two components on cytochrome P450 induction. Hence, a larger sample size such as n = 10-20 may be required to study the interacting effect between tiletamine and zolazepam.

The significance of Telazol®-induced cytochrome P450 induction in field studies remains to be elucidated. The time-course of enzyme induction involved in Telazol®-treatment is presently unknown. A review of the literature indicated that a period of six to eight hours after PB treatment was required for maximal transcriptional activation of cytochrome P450 2B genes (159). Another study showed that a significant increase in cytochrome P450 content occurred starting at 18 hours after treatment of animals with PB at a single dose of 80 mg/kg (160). Hence, it is unlikely that Telazol® will alter enzyme expression within the 0.5 to 4 h period that animals are immobilized in a field study or clinical setting. However, Telazol® may affect the rate of metabolism of other drugs if they are administered to the same animals one to five days after Telazol®-treatment. Hence, it would be useful to determine if the drug, at doses typically employed in the clinic or in the field, induces cytochromes P450 in other species, and to determine the time-course of enzyme induction.

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