DIFFERENTIAL INHIBITION OF HEPATIC CYTOCHROMES P-450
BY CIMETIDINE IN ADULT MALE RATS

By

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ABSTRACT

The cytochrome P-450 enzymes are a family of hemoproteins that play an important role in drug metabolism in man and animals. Cimetidine is a histamine H_2-receptor antagonist used in the treatment of peptic ulcers and other gastric acid-related disorders. It is thought that this drug is a general inhibitor of cytochrome P-450 enzymes. However, a detailed analysis of the literature indicates substantial, but indirect, evidence that certain cytochrome P-450 enzymes may not be inhibited by cimetidine. Also, it is apparent that the observed inhibition of hepatic microsomal cytochrome P-450-mediated enzyme activities by *in vitro* cimetidine administration does not adequately explain the inhibition observed following the *in vivo* administration of the drug to intact animals or to humans.

A major objective of the present study was to determine whether cimetidine, when administered *in vivo*, differentially inhibits cytochrome P-450 enzymes in hepatic microsomes from adult male rats. Uninduced, phenobarbital-induced and dexamethasone-induced rats were sacrificed 90 min after a single intraperitoneal dose of cimetidine HCl (150 mg/kg) or saline. Based on the results from the *in vivo* cimetidine experiments using enzyme-specific substrates and immunoinhibition experiments with monospecific anti-cytochrome P450IIC11 antibody, it was concluded that cimetidine administration to adult male rats inhibited

hepatic cytochrome P450IIC11. Indirect evidence also indicated that unidentified enzymes other than cytochrome P450IIC11 were inhibited by cimetidine in microsomes from uninduced adult male rats. However, the enzyme activities specific for cytochrome P450IIA1, cytochromes P450IIB1/2 and cytochromes P450IIIA1/2 were not affected by *in vivo* cimetidine. It is possible that these enzymes are not inhibited by cimetidine or that the lack of effect is related to the particular substrate used. In some cases, the extent of inhibition of enzyme activities by *in vivo* cimetidine administration depended on prior treatment with an inducer. This can be explained by the increasing contribution to such activities by inducible enzymes which were not subject to inhibition by cimetidine.

Another objective was to determine whether the differential inhibition of cytochrome P-450 by *in vivo* cimetidine is observed when the drug is administered *in vitro*. Cimetidine, at concentrations of up to 10 mM, did not affect the catalytic function of cytochrome P450IIA1. In contrast, it did inhibit enzyme activities that were specific for cytochrome P450IIC11, cytochromes P450IIB1/2 and cytochromes P450IIIA1/2, with IC_{50} values in the range of 1.0 - 7.4 mM. The discrepancy in the inhibition of cytochrome P-450 by *in vivo* and *in vitro* cimetidine administration was further characterized in enzyme kinetic experiments. Based on Lineweaver-Burk plots of the data, the cytochrome P450IIC11-mediated testosterone

2α-hydroxylase activity was inhibited non-competitively by *in vivo* cimetidine, but competitively by *in vitro* cimetidine.

To further investigate the inhibition of cytochrome P-450 enzymes by *in vivo* and *in vitro* cimetidine, preincubation experiments were performed. Hepatic microsomes were preincubated with a low concentration (0.05 mM) of cimetidine and 1 mM NADPH for 15 min prior to the initiation of substrate (testosterone) oxidation. Under these conditions, cimetidine resulted in the inhibition of the enzyme activities specific for cytochrome P450IIC11, but it had no effect on those specific for cytochrome P450IIA1, cytochromes P450IIB1/2 and cytochromes P450IIIA1/2. This differential inhibition by *in vitro* cimetidine required the presence of NADPH in the preincubation medium, suggesting that a catalysis-dependent process is involved. Thus, preincubation of hepatic microsomes with NADPH and a relatively low concentration (0.05 mM) of cimetidine *in vitro* results in a pattern of inhibition of cytochrome P-450 enzymes similar to that following the *in vivo* administration of cimetidine.

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A	androstenedione
APND	aminopyrine N-demethylase
BNF	β-naphthoflavone
cm	centimeter
cyclic AMP	cyclic adenosine 3',5'-monophosphate
DEX	dexamethasone
EDTA	ethylenediaminetetraacetic acid
EMND	erythromycin N-demethylase
EROD	ethoxyresorufin O-deethylase
g	gram
h	hour(s)
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HPLC	high performance liquid chromatography
kg	kilogram
M	molar
mg	milligram
min	minute
mL	milliliter(s)
mm	millimeter
mM	millimolar
NADPH	β-nicotinamide adenine dinucleotide phosphate
nm	nanometers
PB	phenobarbital
PROD	pentoxyresorufin O-dealkylase
TRIS	(Tris[hydroxymethyl]amino-methane)

μg	microgram
μL	microliter
μm	micrometer
μM	micromolar
v/v	volume per unit volume
w/v	weight per unit volume

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INTRODUCTION

1.1 CYTOCHROME P-450

Many drugs are eliminated by metabolism in the liver. Among the hepatic metabolic enzymes are the cytochrome P-450 monooxygenases, a family of closely-related hemoproteins. The prosthetic group in each of these hemoproteins is iron protoporphyrin IX. The term "cytochrome P-450" was coined by Omura and Sato (1964). Carbon monoxide binds to the ferrous form of the hemoprotein and yields a spectral peak at approximately 450 nm. The components of the hepatic cytochrome P-450 enzyme system are: cytochrome P-450, the membrane-bound terminal oxidase which catalyzes substrate oxidation by the addition of an oxygen atom from molecular oxygen; and NADPH-cytochrome P-450 reductase, which reduces cytochrome P-450 by the transfer of two electrons (Ortiz de Montellano, 1986).

Cytochrome P-450 enzymes are found mainly in the liver, but also exist in extrahepatic tissues, including the kidney, lung, brain and intestines (Adesnik and Atchison, 1986). They are most abundant in the endoplasmic reticulum of the cell, which is isolated in the microsomal fraction by differential ultracentrifugation. In addition, these enzymes are also found in the nuclear membrane and mitochondria (Astrom and DePierre, 1986).

Cytochrome P-450 enzymes play a critical role in the oxidative metabolism of exogenous compounds such as drugs and environmental pollutants (Conney, 1982). They also participate in the bioactivation of prodrugs to their pharmacologically active forms (LeBlanc and Waxman, 1989) and in the formation of reactive intermediates that result in toxic, mutagenic and carcinogenic products (Conney, 1982). Cytochrome P-450 enzymes are involved in the biosynthesis and metabolism of endogenous compounds such as steroids, fatty acids and prostaglandins (Kupfer, 1980; Waterman et al., 1986).

1.1.1 Multiplicity

The existence of multiple cytochrome P-450 enzymes had been hypothesized since the initial studies on microsomal drug metabolism. Conney et al., (1959) reported that the administration of benzo[a]pyrene to rats either increased, decreased or had no effect on the hepatic microsomal metabolism of several drugs. Subsequent evidence of broad and overlapping substrate specificity and results from spectral studies led to the conclusion that there were at least two enzymes of cytochrome P-450 in rat livers. The enzyme inducible by phenobarbital and phenobarbital-like inducers was called cytochrome P-450, and the enzyme inducible by 3-methylcholanthrene and polycyclic aromatic hydrocarbon-like inducers was called cytochrome P-448

(Alvares *et al.*, 1967). Data from kinetic studies also provided evidence for the existence of more than one enzyme of cytochrome P-450. Examples include the biphasic decay of radiolabelled cytochrome P-450 heme (Levin *et al.*, 1975) and biphasic Lineweaver-Burk plots of kinetic data from studies of the metabolism of xenobiotics by hepatic microsomes (e.g. Pederson and Aust, 1970). Indeed, since the first report of the partial purification of "cytochrome P-450" and "cytochrome P-448" from rat hepatic microsomes by Lu and Levin (1972), many cytochrome P-450 enzymes have been isolated and purified to apparent homogeneity from several species, including rats, rabbits and man (e.g. Waxman, 1986; Guengerich, 1987, 1989; Ryan and Levin, 1990). It is not known exactly how many cytochrome P-450 enzymes exist, but there may be as many as 200 of them (Renton, 1986).

1.1.2 Nomenclature

The purification of the different cytochrome P-450 enzymes by different investigators has resulted in the development of different nomenclatures. The nomenclatures used by the four major research groups involved in rat hepatic cytochrome P-450 purification are presented in Table 1. The recently recommended gene designation for cytochrome P-450 will be used in this dissertation (Nebert *et al.*, 1989). Each cytochrome P-450 is encoded by a different gene. In this classification system, genes which

TABLE 1

NOMENCLATURE FOR HEPATIC CYTOCHROME P-450 ENZYMES IN RATS

Gene[1] Designation	Guengerich[2]	Levin[3]	Schenkman[4]	Waxman[5]
P450IA1	BNF-B	c	–	BNF-B
P450IA2	ISF-G	d	–	ISF-G
P450IIA1	UT-F	a	RLM2b	3
P450IIA2	–	–	RLM2	–
P450IIB1	PB-B	b	PBRLM5	PB-4
P450IIB2	PB-D	e	PBRLM6	PB-5
P450IIC6	PB-C	k	RLM5a	PB-1
P450IIC7	–	f	RLM5b	–
P450IIC11	UT-A	h	RLM5	2c
P450IIC12	UT-I	i	fRLM4	2d
P450IIC13	–	g	RLM3	–
P450IIE1	–	j	RLM6	–
P450IIIA1	PCN-E	p	–	PB-2a
P450IIIA2	–	–	–	2a
–	–	–	–	6

Due to space restriction, the nomenclature from only the four major groups involved in rat hepatic cytochrome P-450 purification is shown. The recommended gene designation will be used in the thesis.

[1]From: Nebert *et al.*, 1989.
[2]From: Guengerich *et al.*, 1982a; Larrey *et al.*, 1984.
[3]From: Ryan *et al.*, 1979, 1980, 1982a, 1984, 1985; Bandiera *et al.*, 1986; Wrighton *et al.*, 1985a.
[4]From: Cheng and Schenkman, 1982, Backes *et al.*, 1985; Jansson *et al.*, 1985; Favreau *et al.*, 1987.
[5]From: Waxman and Walsh, 1982, 1983; Waxman *et al.*, 1983; Waxman, 1984; Waxman *et al.*, 1988b.

encode proteins that have at least 36% similarity in their amino acid sequences are in the same family. If the similarity is at least 70%, then they are in the same subfamily. In each designation, the Roman numeral indicates the family, the capital letter indicates the subfamily, and the Arabic numeral indicates the gene (see Table 1 for examples). It has been recommended that this system be used when referring to a particular cytochrome P-450 (gene product) (Nebert *et al.*, 1989).

1.1.3 Induction of Cytochrome P-450

Many of the cytochrome P-450 enzymes are subject to induction as a consequence of exposure to xenobiotics or altered physiologic states. The term induction refers to increased *de novo* protein synthesis (Tukey and Johnson, 1990). In some instances, the term is used by investigators to mean increased *de novo* protein synthesis and/or stabilization of existing protein. The major inducible hepatic cytochrome P-450 enzymes in the rat are cytochromes P450IA1, P450IA2, P450IIB1, P450IIB2, P450IIE1, P450IIIA1 and P450IVA1. The preferential inducing agents for these enzymes are shown in Table 2. The level of cytochrome P450IIE1 is also increased in altered physiological states such as diabetes (Bellward *et al.*, 1988) and fasting (Johansson *et al.*, 1988; Ma *et al.*, 1989).

TABLE 2

MAJOR INDUCIBLE HEPATIC CYTOCHROME P-450 ENZYMES IN RATS

Enzyme	Preferential Inducer	References
P450IA1	3-methylcholanthrene	Dannan et al., 1983 Thomas et al., 1983
	β-naphthoflavone	Guengerich et al., 1982a Thomas et al., 1983 Waxman et al., 1985
P450IA2	Isosafrole	Guengerich et al., 1982a Thomas et al., 1983 Waxman et al., 1985
P450IIB1	Phenobarbital	Guengerich et al., 1982a Thomas et al., 1983 Waxman et al., 1985
P450IIB2	Phenobarbital	Guengerich et al., 1982a Thomas et al., 1983 Waxman et al., 1985
P450IIE1	Isoniazid	Thomas et al., 1987
	Ethanol	Thomas et al., 1987
P450IIIA1	Dexamethasone	Heuman et al., 1982
	PCN	Guengerich et al., 1982a Waxman et al., 1985
	TAO	Wrighton et al., 1985a
P450IVA1	Clofibrate	Gibson et al., 1982

Abbreviations: PCN, pregnenolone 16α-carbonitrile;
TAO, triacetyloleandomycin

A characteristic of the inducible cytochrome P-450 enzyme is that one inducer can induce more than one enzyme, and a single enzyme can be induced by many compounds. For example, although phenobarbital preferentially induces cytochromes P450IIB1 and P450IIB2 in rats, it also can induce cytochromes P450IIA1, P450IIC6 and P450IIIA1, although to different extents (Guengerich *et al.*, 1982a; Heuman *et al.*, 1982; Thomas *et al.*, 1983; Waxman *et al.*, 1985). On the other hand, cytochrome P450IIIA1 is inducible in rats by a variety of structurally diverse chemicals, including dexamethasone, pregnenolone 16α-carbonitrile, triacetyloleandomycin, clotrimazole, ketoconazole, and phenobarbital (Guengerich *et al.*, 1982a; Heuman *et al.*, 1982; Waxman *et al.*, 1985; Wrighton *et al.*, 1985a, 1985b; Hostetler *et al.*, 1989).

The mechanism of induction of the different cytochrome P-450 enzymes is still not well understood. However, increased transcription has been shown to occur (Okey, 1990). The induction of cytochromes P450IA1 and P450IA2 by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons requires the binding of the inducer to an endogenous cytosolic receptor (Poland *et al.*, 1976), named the Ah receptor (Okey *et al.*, 1979). The inducer-receptor complex enters the nucleus, and the subsequent interaction between the complex and the nuclear DNA stimulates transcription. Recently, Poland's group purified

the Ah receptor to apparent homogeneity and determined the N-terminal amino acid sequence (Bradfield *et al.*, 1991). It remains to be determined whether receptors are involved in the induction of the other cytochrome P-450 enzymes. It has been shown that transcriptional activation does not occur in the "induction" of cytochrome P450IIE1 in adult rats (Koop and Tierney, 1990). The observed increase in the level of cytochrome P450IIE1 appears to be due to increased stabilization of mRNA, increased translation of synthesized mRNA and/or decreased protein degradation (Koop and Tierney, 1990).

1.1.4 Suppression of Cytochrome P-450

Several of the major cytochrome P-450 enzymes in the uninduced rat have been shown to be resistant to enzyme induction following exposure to known inducing agents. These include cytochrome P450IIA2 (Waxman *et al.*, 1988b), cytochrome P450IIC11 (Guengerich *et al.*, 1982a; Dannan *et al.*, 1983; Waxman, 1984; Waxman *et al.*, 1985; Yeowell *et al.*, 1987, 1989; Emi and Omura, 1988; Shimada *et al.*, 1989), cytochrome P450IIC12 (Waxman *et al.*, 1985) and cytochrome P450IIC13 (Bandiera *et al.*, 1986). The amount (per mg of microsomal protein, per g of liver or percent of total cytochrome P-450) of these enzymes is decreased following the administration of known inducing agents to rats and this effect is called suppression (Guengerich, 1987).

The level of the cytochrome P450IIC11 enzyme in the adult male rat is suppressed following the chronic administration of known inducing agents such as phenobarbital, 3-methylcholanthrene, β-naphthoflavone, dexamethasone and triacetyloleandomycin (Guengerich *et al.*, 1982a, Dannan *et al.*, 1983; Waxman, 1984; Waxman *et al.*, 1985; Yeowell *et al.*, 1987; Miura *et al.*, 1989; Shimada *et al.*, 1989). The level of cytochrome P450IIC11 is also suppressed by the chronic administration of cisplatin (LeBlanc and Waxman, 1988) and cyclophosphamide (LeBlanc and Waxman, 1990). The decline in the level of cytochrome P450IIC11 is accompanied by a decrease in its catalysis of microsomal testosterone 2α-hydroxylation (Waxman, 1984; Yeowell *et al.*, 1987; LeBlanc and Waxman, 1988, 1990; Shimada *et al.*, 1989). It has been well-established that cytochrome P-450-mediated hepatic drug metabolism is impaired following the administration of interferon and interferon inducers to experimental animals (Renton, 1986). Recently, Craig *et al.* (1990) reported that cytochrome P450IIIA2, which is a male-specific cytochrome P-450, is suppressed following the administration of a recombinant interferon or a naturally-derived interferon. Morgan and Norman (1990) reported that the interferon-inducing agents, polyriboinosinic acid, polyribocytidylic acid and R11-877DA, a tilorone analog, suppressed the content of hepatic cytochrome P450IIC11 in adult male rats.

The mechanism of the suppression of cytochrome P-450 enzymes is not known. The decrease in the level of the protein is accompanied by a decrease in the level of the corresponding mRNA. For example, the decline in the content of the cytochrome P450IIC11 protein after the administration of 3-methylcholanthrene, 3,4,5,3′,4′,5′-hexachlorobiphenyl or cyclophosphamide to adult male rats is accompanied by a decrease in the level of cytochrome P450IIC11 mRNA (Yeowell *et al.*, 1987, 1989; LeBlanc and Waxman, 1990). In some instances, multiple mechanisms may be involved. With interferon-inducing agents, both pre- and post-translational mechanisms have been suggested (El Azhary *et al.*, 1980; Morgan and Norman, 1990; Renton *et al.*, 1991).

1.1.5 Inhibition of Cytochrome P-450

The cytochrome P-450 enzymes are subject to inhibition, which is defined in this dissertation as a transient or permanent impairment of the catalytic function of the hemoproteins. This is different from suppression, which is a decrease in the quantity of the hemoprotein. As a result of inhibition, the observed enzyme activity is decreased.

The cytochrome P-450 catalytic cycle is shown in Figure 1. The steps in the cycle are: 1) binding of the substrate to the ferric form of the hemoprotein; 2) electron transfer from NADPH-cytochrome P-450 reductase to produce the ferrous hemoprotein-substrate complex; 3) binding of

Figure 1   The catalytic cycle of cytochrome P-450. (From Murray and Reidy, 1990).
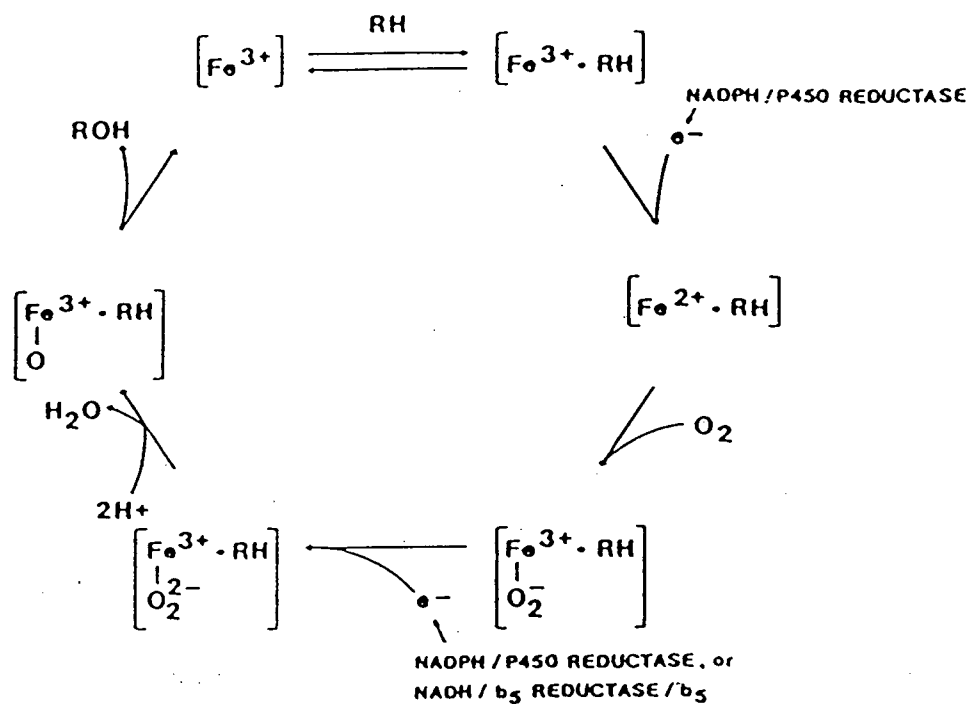
molecular oxygen to the ferrous hemoprotein-substrate complex; 4) reduction of the ferrous hemoprotein-dioxygen complex by the addition of a second electron from NADPH-cytochrome P-450 reductase; 5) drug oxidation by the addition of an oxygen atom from molecular oxygen and reduction of the other oxygen atom to water; and 6) release of the product and recovery of the ferric hemoprotein (Ortiz de Montellano and Reich, 1986). Each step in the catalytic cycle is potentially susceptible to interference.

One approach in classifying the inhibitors of cytochrome P-450 is based on the type of inhibition as determined by enzyme kinetic analysis; for example, competitive, non-competitive or mixed (competitive and non-competitive) inhibition. However, this classification does not identify the mechanism involved (Testa and Jenner, 1981). Non-competitive inhibition can occur as a result of reversible ligand binding by a parent compound (Lesca *et al.*, 1979) or irreversible ligand binding by a metabolite to form a metabolite-intermediate complex (Franklin, 1977). A classification scheme that reflects the mechanism of inhibition is more commonly used. The major mechanisms of inhibition of cytochrome P-450 are reversible inhibition, metabolite-intermediate complexation and mechanism-based inactivation (Testa and Jenner, 1981; Ortiz de Montellano and Reich, 1986; Murray and Reidy, 1990).

1.1.5.1   Reversible Inhibition

Reversible inhibition may occur by substrate (hydrophobic) binding and/or ligand binding. In substrate binding, two compounds compete for the same binding sites on the hemoprotein, resulting in alternate substrate inhibition (Testa and Jenner, 1981). The observed inhibition is competitive and is a function of the relative concentrations of the competing substrates, the lipophilicity of the inhibitors, and the relative affinity of the two compounds for the binding site. In ligand binding, the interaction is between an electron-donating group of a ligand and the ferric or ferrous iron of the hemoprotein (Testa and Jenner, 1981). Oxygen can no longer bind to the heme iron and drug oxidation is impaired. This type of binding depends on the relative affinity of the two competing ligands. Binding occurs with a ligand with a higher affinity for the heme iron. Steric factors are also important in ligand binding. Carbon monoxide is a ligand that can bind to the ferrous hemoprotein and this forms the basis for the standard spectral determination of the total microsomal cytochrome P-450 content (Omura and Sato, 1964).

More commonly, a reversible inhibitor acts by both substrate binding and ligand binding. 9-Hydroxyellipticine inhibits rat hepatic microsomal cytochrome P-450-mediated enzyme activities competitively and non-competitively (Lesca et al., 1979). The competitive inhibition is attributed to

substrate binding, and the non-competitive inhibition to ligand binding (Testa and Jenner, 1981). Other compounds which are thought to inhibit by both substrate binding and ligand binding include metyrapone (Jonen *et al.*, 1974) and the 1-substituted imidazole derivatives such as clotrimazole and ketoconazole (Rodrigues *et al.*, 1987). The inhibitory potency of these inhibitors is determined by the lipophilicity of the inhibitor, affinity of the ligand for the heme iron, and steric hindrance by substituents on the inhibitor (Murray and Ryan, 1983).

1.1.5.2   Metabolite-Intermediate Complexation

A compound can inhibit cytochrome P-450 indirectly by the formation of a metabolite-intermediate complex with the enzyme. In this case, the parent compound may have little or no inhibitory effect. However, once it is oxidized by cytochrome P-450, a metabolite forms a complex with the ferrous and/or ferric hemoprotein by a ligand interaction. As a result, oxygen can not bind to the hemoprotein, rendering the enzyme functionally inactive. The time-dependent formation of a metabolite-intermediate complex occurs both *in vivo* and *in vitro* and proceeds under the same conditions as those required for catalysis by cytochrome P-450. In order for the complex to be generated *in vitro*, the microsomes must be preincubated aerobically with the parent compound and NADPH for a finite period of time.

Several classes of compounds are known to form metabolite-intermediate complexes. These include: 1) the methylenedioxybenzenes such as isosafrole; 2) dioxolanes such as 4-n-butyldioxolane; 3) nitrogenous compounds such as amphetamine, SKF 525-A (diethylaminoethyl-2,2-diphenyl-valerate), propoxyphene, orphenadrine, erythromycin, triacetyloleandomycin and amiodarone; and 4) hydrazines such as N-aminopiperidine (Pershing and Franklin, 1982; Larrey *et al.*, 1986; Reidy *et al.*, 1989).

The inhibition of cytochrome P-450 by metabolite-intermediate complexation has been described as reversible by Testa and Jenner (1981) and irreversible by Ortiz de Montellano and Reich (1986). The complex formed by dioxolane breaks down relatively quickly after formation (Dahl and Hodgson, 1979). In this case, the inhibition is reversible. However, according to Ortiz de Montellano and Reich (1986), with many of the compounds in this class, once the complex is formed *in vivo* or *in vitro*, it is stable and the inhibition that results is irreversible. The degree of stability is demonstrated by the fact that a metabolite-intermediate complex formed *in vivo* remains intact after microsomal preparation. However, the complex formed *in vivo* can be displaced or dissociated *in vitro*. In the ferric state, the metabolite-intermediate-cytochrome P-450 complex formed with a methylendioxybenzene derivative such as isosafrole is unstable (Franklin, 1977). It can be

displaced by lipophilic agents such as cyclohexane (Thomas et al., 1983) or substrates such as 7-ethoxycoumarin (Ryan et al., 1980) and androstenedione (Murray et al., 1986). A nitrogenous compound such as SKF 525-A forms a stable metabolite-intermediate complex with the ferrous iron of the hemoprotein (Buening and Franklin, 1976). This type of complex can be dissociated by potassium ferricyanide, which is an oxidant. Once the complexed cytochrome P-450 has been displaced or dissociated, it is catalytically active. Thus, displacement and dissociation illustrate the quasi-irreversible nature of the binding of the metabolite-intermediate to the iron atom of the hemoprotein (Ortiz de Montellano and Reich, 1986). The mechanism by which a metabolite-intermediate complex is broken down in vivo is not known. A characteristic of the in vivo inhibition of cytochrome P-450 by metabolite-intermediate complex formation is that the inhibition lasts much longer than can be explained by the elimination half-life of the parent compound in the body.

The observed in vitro inhibition of a cytochrome P-450-mediated enzyme activity by a compound that forms a metabolite-intermediate complex can be competitive, non-competitive or mixed (competitive and non-competitive), depending on the experimental conditions. Without the aerobic preincubation of microsomes with the parent compound and NADPH, the inhibition of the enzyme activity can be

competitive. This is attributed to alternate substrate inhibition by the parent compound (Franklin, 1977; Testa and Jenner, 1981). However, with preincubation, either non-competitive or mixed (competitive and non-competitive) inhibition may be observed, depending on the concentrations of the parent compound used in a given experiment (Franklin, 1977). At low concentrations, non-competitive inhibition is observed and this is due to the ligand binding of a metabolite to the heme iron (Franklin, 1977; Testa and Jenner, 1981). At high concentrations, both competitive and non-competitive inhibition occurs and this is due to both ligand binding by a metabolite and alternate substrate inhibition by the parent compound (Franklin, 1977; Testa and Jenner, 1981).

The characteristics of a metabolite-intermediate complex are as follows. First, complex formation is a time-dependent process. Second, the complex shows a spectral peak at 448-456 nm, depending on the particular compound. This allows for the formation of the complex to be observed *in vitro*. Third, the complexed cytochrome P-450 is catalytically inactive, whereas the uncomplexed form is catalytically active. Fourth, the complexed cytochrome P-450 can not be measured spectrally by the standard dithionite-reduced carbon monoxide-binding method of Omura and Sato (1964). Therefore, in order to measure the total (complexed and uncomplexed) cytochrome P-450 content, the

metabolite-intermediate complex must first be dissociated or displaced.

1.1.5.3   Mechanism-Based Inactivation

Mechanism-based inactivation is an enzyme-mediated and irreversible process (Rando, 1984).   The first step in mechanism-based inactivation is the formation of a reactive intermediate from the parent compound by a cytochrome P-450 enzyme.   The second step involves the inactivation of that enzyme by the reactive intermediate (Ortiz de Montellano, 1988).   Therefore, the enzyme initiates its own destruction. An agent which inhibits a cytochrome P-450 in this manner is called a mechanism-based inactivator (Rando, 1984) or a suicide substrate (Walsh, 1982).

Mechanism-based inactivation occurs as a result of covalent binding of a reactive intermediate to the prosthetic heme group or the apoprotein of a cytochrome P-450 enzyme (Ortiz de Montellano and Reich, 1986).   Agents that form reactive intermediates which bind covalently to the prosthetic heme group of cytochrome P-450 include: terminal olefins such as allylisopropylacetamide and secobarbital; terminal acetylenes such as ethinyl estradiol and danazol; and heterocyclic compounds such as 1-aminobenzotriazole and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (Ortiz de Montellano and Correia, 1983).   In these cases, enzyme inactivation occurs as a

result of the N-alkylation of the prosthetic heme group by a reactive intermediate. The same molar amount of heme and apoprotein is lost and the alkylated heme moiety can be isolated. Agents that form reactive intermediates which bind covalently to the apoprotein of cytochrome P-450 include: halogenated compounds such as chloramphenicol; and sulfur-containing compounds such as parathion and carbon disulfide (Ortiz de Montellano and Reich, 1986). The detailed mechanism of enzyme inactivation by many of these compounds is not well known. However, in the case of chloramphenicol, the oxamyl intermediate of chloramphenicol formed by cytochrome P-450 acylates a lysine residue in the active center of the protein (Halpert, 1981). This modification impairs the transfer of electrons from NADPH-cytochrome P-450 reductase to cytochrome P-450 (Halpert *et al.*, 1985b) and substrate (drug) oxidation is impaired.

The observed *in vitro* inhibitory effect of a mechanism-based inactivator on cytochrome P-450-mediated enzyme activities depends on the experimental conditions. Both reversible and irreversible inhibition have been observed with chloramphenicol when it is added *in vitro* to microsomes. Reversible inhibition is observed when chloramphenicol is added immediately prior to the initiation of the enzymatic reaction and this is due to alternate substrate inhibition by the parent compound (Grogan *et al.*, 1972; Reilly and Ivey, 1979). Irreversible inhibition is

observed when chloramphenicol is preincubated with the microsomes prior to the initiation of substrate oxidation (Halpert *et al.*, 1983). The preincubation allows for the formation of the reactive metabolite that binds covalently to the apoprotein of cytochrome P-450 (Halpert, 1981). Irreversible inhibition of cytochrome P-450-mediated enzyme activities is also observed when chloramphenicol is administered *in vivo* to rats (Halpert *et al.*, 1983, 1985a).

1.1.5.4 Single vs. Multiple Doses of an Inhibitor

A compound may act as an inhibitor and an inducer of cytochrome P-450, depending on the duration of treatment. SKF 525-A and triacetyloleandomycin inhibit cytochrome P-450-mediated enzyme activities following a single injection to rats, with the major mechanism being metabolite-intermediate complexation (Buening and Franklin, 1976; Pessayre *et al.*, 1981). However, after multiple injections of these agents over several days, the level of cytochrome P-450 enzymes and their catalytic function are increased (e.g. Schenkman *et al.*, 1972; Buening and Franklin, 1976; Wrighton *et al.*, 1985a; Murray, 1988). The full extent of these increases is observed following dissociation or displacement of the metabolite-intermediate complex *in vitro*. The observed effect with multiple dosing is due to both increased *de novo* protein synthesis and decreased degradation of existing proteins (Watkins *et al.*,

1986). Therefore, in experiments to study the *in vivo* inhibitory action, the compound should be administered as a single dose to avoid this complication, unless the intent is to increase the amount of the enzyme present as an enzyme-metabolite-intermediate complex.

1.1.5.5   Selective Inhibition of Cytochrome P-450

Cytochrome P-450 enzyme activities appear to be subject to differential inhibition. For example, 9-hydroxy-ellipticine inhibits ethoxyresorufin O-deethylase activity in microsomes from rats pretreated with 3-methylcholan-threne, but not ethylmorphine N-demethylase activity in microsomes from rats pretreated with phenobarbital (Phillipson *et al.*, 1985). Metyrapone is more potent in inhibiting p-nitroanisole O-demethylase activity than aniline hydroxylase activity in microsomes from rats pretreated with phenobarbital (Jonen *et al.*, 1974).

An aspect of research in the field of cytochrome P-450 has been the design and synthesis of selective inhibitors. Selective cytochrome P-450 enzyme inhibition has been shown with several compounds, including certain derivatives of 1-aminobenzotriazole (Mathews and Bend, 1986), chloramphenicol (Stevens and Halpert, 1988; Halpert *et al.*, 1990), progesterone (Halpert *et al.*, 1989a), pregnenolone (Halpert *et al.*, 1989b) and 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (Riddick *et al.*, 1990). The

identification of relatively selective inhibitors has been facilitated by the use of enzyme-specific substrates. The most powerful approach involves the use of one of the following substrates: testosterone, androstenedione, progesterone or warfarin (Kaminsky et al., 1979; Waxman, 1988). These compounds are hydroxylated in a regioselective and stereoselective manner. An advantage of using one of these compounds as a substrate is that, in some instances, a particular cytochrome P-450 is the major or sole catalyst in the formation of a metabolite. Another advantage is that the multiple metabolites formed by the same microsomal preparation can be analyzed simultaneously. To date, not one inhibitor has been shown to be enzyme-specific; that is, one which inhibits only a single enzyme. An enzyme-specific inhibitor used in conjunction with an enzyme-specific substrate would be very useful in studying the function of a particular cytochrome P-450.

1.1.6 Specificity of Microsomal Enzyme Activities

The role of a cytochrome P-450 enzyme in the metabolism of a substrate can be determined in microsomes by immunoinhibition experiments. At saturating concentrations of a monospecific antibody preparation, the contribution of a given enzyme to a substrate reaction can be estimated by determining the percent inhibition of the enzyme activity by the antibody. The purification of cytochrome P-450 enzymes

and the preparation of monospecific antibodies have facilitated the identification of enzyme activities that are specific for particular cytochrome P-450 enzymes. To date, several specific microsomal enzyme activities have been identified using this approach. Some of these are shown in Table 3.

The specificity of an enzyme activity for a cytochrome P-450 enzyme depends on factors such as prior drug treatment and the type of tissue under examination. Cytochromes P450IIB1/2 account for more than 90% of the pentoxyresorufin O-dealkylase activity in hepatic microsomes from phenobarbital-treated rats (Lubet et al., 1985; Waxman et al., 1987; Dutton and Parkinson, 1989), but do not contribute to this activity in microsomes from uninduced rats (Waxman et al., 1987). Testosterone 16α-hydroxylase activity is specific for cytochrome P450IIC11 in hepatic microsomes from uninduced rats (Waxman, 1984; Waxman, 1987), but not in microsomes from phenobarbital-treated rats (Thomas et al., 1981; Reik et al., 1985; Waxman et al., 1987). Recently, Sesardic et al. (1990a) demonstrated that the high-affinity phenacetin O-deethylase activity was specific for cytochrome P450IA2 in microsomes prepared from the liver, kidney and gut, but not those from the lung. These examples illustrate the importance of and need for verifying the specificity of a given cytochrome P-450-

TABLE 3

CONTRIBUTION OF RAT HEPATIC CYTOCHROME P-450 TO MICROSOMAL ENZYME ACTIVITIES

Enzyme Activity	Inducer Treatment	%	Enzyme	Reference
Erythromycin N-Demethylase	DEX	55-60	P450IIIA1/2	Wrighton et al., 1985a
Ethoxyresorufin 0-Deethylase	3MC	82	P450IA1	Kelley et al., 1987
	3MC	> 90	P450IA1	Dutton and Parkinson, 1989
	None	6	P450IA1	Kelley et al., 1987
	3MC	27	P450IA2	Kelley et al., 1987
	None	78	P450IA2	Kelley et al., 1987
Pentoxyresorufin 0-Dealkylase	PB	> 90	P450IIB1/2	Lubet et al., 1985
	PB	> 90	P450IIB1/2	Waxman et al., 1987
	PB	> 90	P450IIB1/2	Dutton and Parkinson, 1989
	None	0	P450IIB1/2	Waxman et al., 1987
Testosterone Hydroxylase				
2α	None	> 85	P450IIC11	Waxman, 1984 Waxman et al., 1987
2β, 6β	None	> 85	P450IIIA1/2	Halvorson et al., 1990
	PB	> 85	P450IIIA1/2	Halvorson et al., 1990
	DEX	> 85	P450IIIA1/2	Halvorson et al., 1990
7α	None	> 97	P450IIA1	Levin et al., 1987
		80	P450IIA1	Waxman et al., 1988b
	PB	> 98	P450IIA1	Arlotto and Parkinson, 1989
	DEX	> 96	P450IIA1	Arlotto and Parkinson, 1989
		> 98	P450IIA1	Levin et al., 1987
		> 96	P450IIA1	Arlotto and Parkinson, 1989
16α	None	> 85	P450IIC11	Waxman, 1984 Waxman et al., 1987
	None	14	P450IIB1/2	Thomas et al., 1981
		0	P450IIB1/2	Reik et al., 1985
		0	P450IIB1/2	Waxman et al., 1987
	PB	66	P450IIB1/2	Thomas et al., 1981
		77	P450IIB1/2	Reik et al., 1985
		60-70	P450IIB1/2	Waxman et al., 1987
	PB	30	P450IIC11	Waxman, 1984 Waxman et al., 1987
16β	PB	89	P450IIB1/2	Reik et al., 1985
	PB	> 90	P450IIB1/2	Waxman et al., 1987

Abbreviations: PB, phenobarbital; DEX, dexamethasone; 3MC, 3-methylcholanthrene

mediated microsomal enzyme activity under the experimental
conditions.

1.2 CIMETIDINE

1.2.1 Chemical Structure

Cimetidine is a 4,5-substituted imidazole derivative with a cyano group attached to its side chain. Its chemical structure is shown in Figure 2.

1.2.2 Pharmacology

The basolateral membrane of the gastric mucosal and parietal cells contain receptors for histamine, gastrins and acetylcholine. These particular receptors for histamine are called histamine H_2-receptors (Black *et al.*, 1972). Stimulation of histamine H_2-receptors activates adenylate cyclase, which in turn increases the intracellular concentration of cyclic AMP (Hill, 1990). As a result, specific cyclic AMP-dependent protein kinases are activated, the proton pump, a H^+,K^+-ATPase, is stimulated, and the secretion of hydrogen ions into the stomach is increased (Wolfe and Soll, 1988). It has been shown that stimulation of the receptors for gastrins and acetylcholine also results in the secretion of hydrogen ions (Hill, 1990).

Cimetidine is a competitive histamine H_2-receptor antagonist (Brimblecombe *et al.*, 1975). As result of histamine H_2-receptor blockade by cimetidine, both the basal

Figure 2 The metabolic pathways for cimetidine in rats. (From: Taylor *et al.*, 1978; Zbaida *et al.*, 1984)
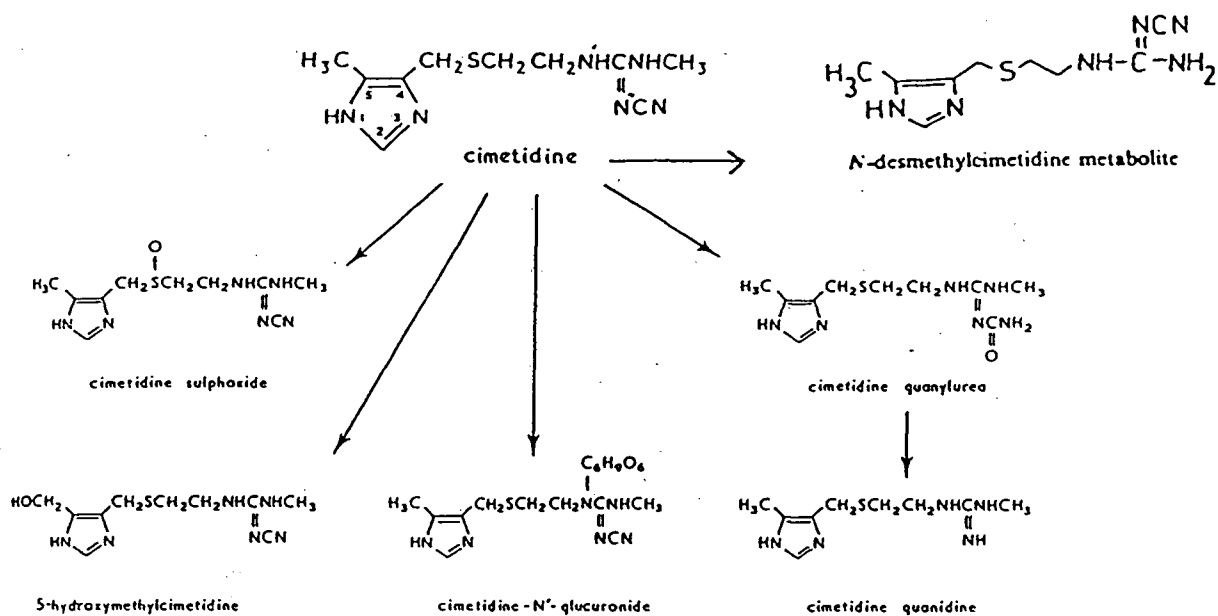
and stimulated secretion of gastric acid are reduced (Pounder, 1984).

In 1977, cimetidine was approved for clinical use in Canada. It is used therapeutically in the treatment of peptic ulcers and other gastric acid-related disorders (Feldman and Burton, 1990b).

1.2.3 Pharmacokinetics

Cimetidine is rapidly absorbed after oral administration in man. Following the ingestion of a 400 mg tablet of cimetidine, the peak serum concentration is approximately 10 μM and occurs at 90 min after dosing (Griffiths *et al.*, 1977). The absolute bioavailability after an oral dose ranges from 58-89% (Lin, 1991). In rats, more than 90% of an oral dose is absorbed (Taylor *et al.*, 1978).

In man, cimetidine is distributed to the kidney, gallbladder, stomach, liver and skeletal muscle (Schentag *et al.*, 1981). In addition, cimetidine penetrates the blood-brain barrier and distributes into the cerebrospinal fluid (Jonsson *et al.*, 1982). The distribution of cimetidine in rats is similar to that in man except that there is a lack of penetration into the central nervous system (Cross, 1977). Cimetidine in plasma is only 13-25% bound to plasma proteins in man (Taylor *et al.*, 1978; Somogyi *et al.*, 1980)

and 10-23% in rats (Taylor *et al.*, 1978; Adedoyin *et al.*, 1987a).

The elimination half-life of cimetidine is approximately 2 h in healthy human volunteers and ulcer patients (Somogyi and Gugler, 1983) and 30-45 min in rats (Weiner and Roth, 1981; Adedoyin *et al.*, 1987a).

Cimetidine is eliminated mainly by renal tubular secretion and glomerular filtration (Somogyi *et al.*, 1980; Weiner and Roth, 1981). In a 24 h period following the ingestion of a single oral dose of cimetidine in man, 70-80% of the dose is recovered in urine (Burland *et al.*, 1975; Taylor *et al.*, 1978; Mitchell *et al.*, 1982). In rats, the recovery is 60-70% in the same period (Taylor *et al.*, 1978). In both cases, 50-75% of the total recovery is the parent compound (Taylor *et al.*, 1978; Mitchell *et al.*, 1982; Dixon *et al.*, 1985; Adedoyin *et al.*, 1987a).

The metabolism of cimetidine in man and rats involves glucuronidation, oxidation and hydrolysis (Figure 2). The most abundant metabolite of cimetidine in man is cimetidine-N'-glucuronide, which accounts for 24% of the total urinary excretion in the first 24 h after dosing (Mitchell *et al.*, 1982). The other metabolites of cimetidine are cimetidine sulfoxide (7-19%), 5-hydroxymethylcimetidine (4-5%), cimetidine guanylurea (2%), and cimetidine guanidine (<0.1%) (Burland *et al.*, 1975; Griffiths *et al.*, 1977; Taylor *et al.*, 1978; Mitchell *et al.*, 1982). In rats, cimetidine

sulfoxide, 5-hydroxymethylcimetidine and N-desmethyl-cimetidine have been shown to be generated from cimetidine by rat hepatic microsomes (Zbaida *et al.*, 1984).

1.2.4 Cimetidine Drug-Drug Interactions

Since the first publication of a controlled clinical study of a cimetidine drug interaction with warfarin in 1979 (Serlin *et al.*, 1979), numerous other drug-drug interactions involving cimetidine have been identified. In many of these cases, the interaction is due to inhibition of hepatic drug metabolism by cimetidine. The topic of cimetidine drug-drug interactions in man has been reviewed extensively in the literature (e.g. Somogyi and Muirhead, 1987; Smith and Kendall, 1988; Feldman and Burton, 1990a). In man, cimetidine impairs the clearance of drugs that undergo extensive cytochrome P-450-mediated hepatic oxidative metabolism, but does not affect the clearance of drugs that are mainly eliminated by conjugation reactions such as glucuronidation, sulphation and acetylation. In animal studies, the *in vitro* addition of cimetidine to hepatic microsomes has been shown to inhibit the cytochrome P-450-catalyzed oxidation of many substrates (Table 4). Observations from human and animal studies have led to the perception that cimetidine is a general inhibitor of cytochrome P-450 (Reilly *et al.*, 1988; Leclercq *et al.*, 1989). However, a detailed analysis of the literature

TABLE 4

INHIBITION OF HEPATIC MICROSOMAL CYTOCHROME P-450 ENZYME
ACTIVITIES BY *IN VITRO* CIMETIDINE IN RATS

Enzyme Activity	Type of Inhibition	Ki (mM)	Reference
aminopyrine N-demethylase	non-competitive	N.D.	Pelkonen and Puurunen, 1980
aminopyrine N-demethylase	non-competitive	N.D.	Tanaka *et al.*, 1985
aminopyrine N-demethylase	mixed	0.13	Speeg *et al.*, 1982
aminopyrine N-demethylase	mixed	0.7	Imai *et al.*, 1986
benzo[a]pyrene hydroxylase	non-competitive	N.D.	Pelkonen and Puurunen, 1980
7-ethoxycoumarin 0-deethylase	competitive	0.18 0.3	Rendic *et al.*, 1979
7-ethoxycoumarin 0-deethylase	competitive	0.8	Jensen and Gugler, 1985
meperidine N-demethylase	competitive	0.45	Knodell *et al.*, 1982
meperidine N-demethylase	non-competitive	N.D.	Dawson and Vestal, 1984
metoprolol α-hydroxylase	competitive	0.009	Lennard *et al.*, 1986
metoprolol α-hydroxylase	competitive	0.019	Wright *et al.*, 1991
metoprolol 0-desmethylase	competitive	0.038	Lennard *et al.*, 1986
morphine N-demethylase	competitive	0.068	Reilly and Winzor, 1984
pentobarbital hydroxylase	competitive	0.13	Knodell *et al.*, 1982
trimethadione N-demethylase	non-competitive	N.D.	Tanaka *et al.*, 1985

N.D. = not determined

indicated substantial, but indirect, evidence that certain cytochrome P-450 enzymes may be less susceptible or even refractory to the inhibitory action of cimetidine.

1.2.5 Differential Inhibition of Cytochrome P-450-Mediated Hepatic Drug Metabolism by *In Vivo* Cimetidine Treatment

1.2.5.1 Clinical Studies

It has been shown in clinical studies that cimetidine does not affect the clearance or steady-state serum concentrations of several drugs that are metabolized mainly by cytochrome P-450 enzymes. The results of these studies are summarized in Table 5. A few of these will be explained in detail.

Tolbutamide is metabolized in man to hydroxytolbutamide, which is then partially converted to carboxytolbutamide (Thomas and Ikeda, 1966). Various drugs have been shown to affect the clearance of tolbutamide, including sulphaphenazole (Hansen and Christensen, 1977). The administration of cimetidine to healthy human volunteers has no effect on the total body clearance of tolbutamide (Dey *et al.*, 1983; Stockley *et al.*, 1986; Adebayo and Coker, 1988) or the formation clearance of the hydroxytolbutamide metabolite (Stockley *et al.*, 1986). These observations suggest that the cytochrome P-450 enzyme(s) involved in the hydroxylation of tolbutamide is(are) not subject to

TABLE 5

CLINICAL STUDIES WITH CIMETIDINE:
LACK OF A DRUG-DRUG INTERACTION

Drug	Variable Not Affected by Cimetidine	N	Reference
carbamazepine	steady-state serum concentration*	7 / 11 / 8	Sonne et al., 1983 / Levine et al., 1985 / Dalton et al., 1986
cyclosporine	total body clearance	2	Jarewenko et al., 1986
desipramine	total body clearance**	4	Steiner and Spina, 1987
	formation clearance of 2-hydroxy-desipramine**	4	Steiner and Spina, 1987
estradiol	16α-hydroxylation of estradiol in serum	9	Galbraith and Michnovicz, 1989
	urinary excretion of 16α-hydroxy-estrone	9	Galbraith and Michnovicz, 1989
mexiletine	total body clearance	6 / 6	Klein et al., 1985 / Brockmeyer et al., 1989
	formation clearance of 4-hydroxymethyl-mexiletine	6	Brockmeyer et al., 1989
	formation clearance of para-hydroxymethy-mexiletine	6	Brockmeyer et al., 1989
misonidazole	total body clearance	6	Begg et al., 1983
	AUC for 0-desmethyl-misonidazole	6	Begg et al., 1983
tolbutamide	total body clearance	10 / 7 / 8 / 7	Dey et al., 1983 / Stockley et al., 1986 / Adebayo et al., 1988 / Stockely et al., 1986
	formation clearance of hydroxytolbutamide		

N = number of subjects; AUC = area under the serum-concentration curve
* In patients on chronic carbamazapine therapy
** In slow metabolizers of debrisoquine

inhibition by cimetidine. Recently, it was reported that at least three human cytochrome P-450 enzymes are involved in the hydroxylation of tolbutamide: cytochrome P450IIC8, cytochrome P450IIC9 and cytochrome P450IIC10 (Brian *et al.*, 1989; Relling *et al.*, 1990).

The enzyme involved in the hydroxylation of debrisoquine to 4-hydroxydebrisoquine exhibits genetic polymorphism and two distinct phenotypes have been noted: extensive and poor metabolizers (Mahgoub *et al.*, 1977). The enzyme which hydroxylates debrisoquine in man has been purified (Gut *et al.*, 1984; Distlerath *et al.*, 1985) and is referred to as cytochrome P450IID6 (Nebert *et al.*, 1989). The oxidative metabolism of some drugs, including desipramine, has been associated with the polymorphism of debrisoquine hydroxylation (Bertilsson and Aberg-Wistedt, 1983). The concurrent administration of cimetidine and desipramine to healthy human volunteers results in a decrease in the total body clearance of desipramine and a decrease in the formation clearance of the 2-hydroxy-desipramine metabolite in extensive metabolizers of debrisoquine (Steiner and Spina, 1987). In contrast, in poor metabolizers of debrisoquine, cimetidine does not affect the total body clearance of desipramine or the formation clearance of the 2-hydroxydesipramine metabolite (Steiner and Spina, 1987). It has been shown that there is no immunodetectable cytochrome P450IID6 in hepatic

microsomes from poor metabolizers of debrisoquine (Gonzalez et al., 1988; Zanger et al., 1988). In poor metabolizers, other cytochrome P-450 enzyme(s) are likely to be involved in the oxidation of debrisoquine and other drugs associated with the polymorphism of debrisoquine hydroxylation. The results from the cimetidine-desipramine drug interaction study therefore suggest that cimetidine inhibits cytochrome P450IID6, but not the cytochrome P-450 enzyme(s) involved in the hydroxylation of desipramine in poor metabolizers.

Carbamazepine is eliminated mainly by hepatic metabolism (Lertratanangkoon and Horning, 1982). Cimetidine decreases the total body clearance of carbamazepine in healthy human volunteers administered a single dose of carbamazepine (Webster et al., 1984; Dalton et al., 1985). In contrast, in epileptic patients on chronic carbamazepine therapy (Sonne et al., 1983; Levine et al., 1985) and in healthy human volunteers pretreated with multiple doses of carbamazepine (Dalton et al., 1986), cimetidine has no effect on the steady-state serum concentration of carbamazepine. Carbamazepine is an inducer of cytochrome P-450 and it induces its own metabolism (Eichelbaum et al., 1975, 1985). It is therefore possible that cimetidine inhibits the enzyme(s) involved in carbamazepine metabolism in uninduced subjects, but not the enzyme(s) involved in the metabolism of this drug in subjects undergoing chronic carbamazepine therapy.

Antipyrine is used frequently as an *in vivo* marker for cytochrome P-450-mediated hepatic drug metabolism in man (Vesell, 1979). Feely *et al.* (1984) demonstrated that the percent decrease in antipyrine clearance by cimetidine was almost two-fold greater in subjects pretreated with rifampin than in subjects who were not pretreated. Rifampin is an efficacious inducer of human cytochrome P450IIIA enzymes (Combalbert *et al.*, 1989). It is possible that the cytochrome P-450 enzyme(s) responsible for antipyrine oxidation in the rifampin-induced subjects is(are) more susceptible to inhibition by cimetidine than those involved in the metabolism of antipyrine in uninduced subjects.

1.2.5.2 Animal Studies

Antipyrine is eliminated in rats by hepatic metabolism to yield three major metabolites: 3-hydroxymethylantipyrine, 4-antipyrine and norantipyrine (Danhof *et al.*, 1979). Multiple cytochrome P-450 enzymes are thought to be involved in the formation of the metabolites of antipyrine. The *in vivo* administration of cimetidine to adult male rats differentially inhibits the *in vivo* formation of the major metabolites of antipyrine (Adedoyin *et al.*, 1987b). Cimetidine is approximately 50 times more potent in inhibiting the formation of 3-hydroxymethylantipyrine than in inhibiting the formation of 4-hydroxyantipyrine (Adedoyin *et al.*, 1987b). This suggests that the formation of the

metabolites of antipyrine is catalyzed by different cytochrome P-450 enzymes and that these enzymes have differential susceptibility to inhibition by cimetidine.

Drew *et al.* (1981) reported that a single intraperitoneal administration of cimetidine (150 mg/kg) to adult male rats pretreated with 3-methylcholanthrene resulted in 89% inhibition of hepatic microsomal benzo[a]pyrene hydroxylase activity, but had no effect on 7-ethoxycoumarin O-deethylase, biphenyl 4-hydroxylase, zoxazolamine hydroxylase or aniline hydroxylase activity. This suggests that certain cytochrome P-450 enzymes are not susceptible to inhibition by *in vivo* cimetidine treatment in adult male rats.

Recently, Galbraith and Jellinck (1989) demonstrated that multiple intraperitoneal injections of cimetidine (173 mg/kg every 12 hours for 5 doses) to adult male rats resulted in a decrease in hepatic microsomal estradiol 2-hydroxylase, estradiol 16α-hydroxylase, ethylmorphine N-demethylase, aniline hydroxylase and benzo[a]pyrene hydroxylase activities, but had no effect on 7-ethoxycoumarin O-deethylase activity. In contrast, none of these activities were affected in hepatic microsomes from adult female rats subjected to the same cimetidine treatment protocol. These results are difficult to interpret since cimetidine was administered as multiple injections over several days rather than as a single dose. It has been

shown that treatment of adult male rats with multiple injections of cimetidine modestly induces hepatic cytochromes P450IA1/2 and cytochromes P450IIB1/2 (Ioannides et al., 1989). After repeated administration of cimetidine over several days, the relative proportions of various cytochrome P-450 enzymes in the hepatic microsomes may have been changed, due to induction of some enzymes and suppression of others. Thus, a decrease in an enzyme activity under these circumstances may be due not only to inhibition, but also to suppression. However, if the observations by Galbraith and Jellinck (1989) were mainly a result of inhibition, then they would suggest that certain cytochrome P-450 enzymes in adult male and female rats are not susceptible to the inhibitory action of cimetidine.

Taken together, one interpretation from the human and animal studies with cimetidine described above is that not all cytochrome P-450 enzymes are inhibited following in vivo cimetidine treatment. To date, systematic studies have not been performed to determine whether cimetidine selectively inhibits cytochrome P-450 enzymes. The information generated from such studies would be important for several reasons. First, in order to develop a detailed understanding of the inhibition of hepatic drug metabolism by cimetidine, it is necessary to initially identify the specific cytochrome P-450 enzymes inhibited by the compound before any mechanistic studies can be performed. Second, in

certain instances, the information generated may help to predict potential drug-drug interactions with cimetidine. Third, knowledge of the specific enzyme(s) inhibited by cimetidine would allow investigators to use this compound as a pharmacological probe in cytochrome P-450 research; for example, to study the function of a particular enzyme.

1.2.6 Inhibition of Cytochrome P-450 by *In Vitro* or *In Vivo* Cimetidine Treatment

Inhibition of cytochrome P-450 enzyme activities by the *in vitro* addition of cimetidine to microsomes has been well documented. Cimetidine interacts with rat and human microsomal cytochrome P-450 *in vitro* by the binding of a ligand nitrogen atom to the heme iron of the hemoprotein at the sixth coordination position, resulting in a characteristic Type II difference spectrum with the peak and trough at 420-432 nm and 390-397 nm, respectively (Rendic *et al.*, 1979, 1983, 1984; Pelkonen and Puurunen, 1980; Speeg *et al.*, 1982; Knodell *et al.*, 1982; Bast *et al.*, 1989). At present, the mechanism by which cytochrome P-450 enzymes are inhibited following *in vivo* cimetidine administration is still not known. However, it has become apparent that the observed inhibition of cytochrome P-450 by *in vitro* cimetidine is not equivalent to the effect observed following the *in vivo* administration of the drug to intact animals or to humans (Somogyi and Muirhead, 1987).

The concentration of cimetidine required for the *in vitro* inhibition of a microsomal cytochrome P-450-mediated enzyme activity is typically 100-1000 times greater than the serum concentration associated with inhibition of drug metabolism *in vivo*. In *in vitro* enzyme inhibition studies, the IC_{50} is the concentration of the inhibitor required to reduce an enzyme activity by 50%. In rat hepatic microsomes, the usual IC_{50} for inhibition of various enzyme activities by *in vitro* cimetidine is in the range of 1-10 mM (Pelkonen and Puurunen, 1980; Speeg *et al.*, 1982; Dawson and Vestal, 1984; Mosca *et al.*, 1985; Imai *et al.*, 1986; Yee and Shargel, 1986; Wang *et al.*, 1988; Bast *et al.*, 1989; Vyas *et al.*, 1990). In contrast, at 1 h after a single intraperitoneal dose (120 mg/kg) of cimetidine to rats, substantial inhibition of aminopyrine elimination is observed, whereas the serum drug concentration is only approximately 0.008 mM (Speeg *et al.*, 1982). In human hepatic microsomes, the IC_{50} for inhibition of cytochrome P-450 enzyme activity by *in vitro* cimetidine is also in the range of 1-10 mM (Puurunen *et al.*, 1980, Rendic *et al.*, 1984; Hoensch *et al.*, 1985; Imai *et al.*, 1986; Pasanen *et al.*, 1988; Vyas *et al.*, 1990). During chronic administration of cimetidine at therapeutic doses in man, the serum concentration of the drug is below 0.006 mM for most of the dosage interval (Somogyi and Gugler, 1983) and

inhibition of drug metabolism is known to occur at these low serum concentrations (Cohen *et al.*, 1985).

To reconcile the differences between the inhibition of cytochrome P-450 by *in vitro* and *in vivo* cimetidine, several interpretations have been made by various investigators.

Based on data from spectral binding studies, it has been suggested that at least two cimetidine-binding sites exist in rat hepatic microsomes, with spectral dissociation constants (K_s) of 0.008 - 0.072 mM and 0.10 - 0.33 mM (Rendic *et al.*, 1979, 1983; Speeg *et al.*, 1982; Reilly *et al.*, 1983; Rekka *et al.*, 1988). To account for the discrepancy between the concentrations required for inhibition by *in vivo* and *in vitro* cimetidine, Reilly *et al.* (1983) proposed that the inhibition observed with *in vivo* cimetidine is due to its interaction with the higher affinity binding site. A major problem with this proposal is that the spectral binding studies with cimetidine were all performed with microsomes and not with a purified cytochrome P-450. The observation of biphasic binding may simply reflect the different affinities of the various cytochrome P-450 enzymes in the microsomes for cimetidine.

The IC_{50} for *in vitro* inhibition is dependent on the type of inhibition. In the case of competitive inhibition, $IC_{50} = Ki (1 + [S] / Km)$, where Ki is the inhibitory constant, [S] is the substrate concentration and Km is the Michaelis constant. Reilly and Winzor (1984) suggested that

the high IC_{50} values observed in various *in vitro* cimetidine studies may simply be due to the high substrate concentrations usually used in the assays. The value of IC_{50} is dependent on the ratio of [S] / Km if the inhibition is competitive. It has been documented that the inhibition of cytochrome P-450 enzyme activities by *in vitro* cimetidine is not always competitive (Table 4). Moreover, substantial inhibition of microsomal cytochrome P-450-mediated enzyme activities is observed 2 h after a single intraperitoneal injection of cimetidine to rats (Drew *et al.*, 1981).

The standard method for performing *in vitro* inhibition studies involves the addition of the putative inhibitor to the incubation mixture immediately prior to the initiation of substrate oxidation. However, this method does not allow for the catalysis-dependent formation of reactive intermediates. Jensen and Gugler (1985) therefore used a preincubation protocol in an attempt to further investigate the inhibition of cytochrome P-450 by cimetidine in rats. They observed that a 10 min preincubation of microsomes with cimetidine (0.25 mM) and NADPH prior to the addition of the substrate increased the inhibition of 7-ethoxycoumarin 0-deethylase activity compared to "control" samples not subjected to preincubation. Based on this observation, these investigators proposed that the inhibition of cytochrome P-450 by cimetidine *in vivo* involves the formation of a metabolite-intermediate or an activated

complex. However, the increase in the inhibition of the enzyme activity reported by Jensen and Gugler (1985) following preincubation may have been due to the presence of NADPH in the preincubation mixture. It has been shown that lipid peroxidation by rat hepatic microsomes is NADPH-dependent and that this results in the breakdown of heme from the holoenzyme (Levin et al., 1973). Ioannoni et al. (1986) claimed that when the effect by NADPH was taken into account, preincubation had no effect on the inhibition of microsomal morphine N-demethylation by 0.5 mM cimetidine. According to Rekka et al. (1988), preincubation of hepatic microsomes with cimetidine (0.1 mM or 0.25 mM) did not increase the inhibition of the microsomal oxidation of tofenacine or 7-ethoxyresorufin. However, they failed to specify whether the control samples were preincubated with NADPH. It is possible that cimetidine forms a metabolite-intermediate complex only with certain cytochrome P-450 enzymes. To date, it has not been shown conclusively whether metabolite-intermediate complexation is a mechanism involved in the inhibition of cytochrome P-450 enzymes by cimetidine.

It is apparent that the standard method for conducting inhibition studies with cimetidine added *in vitro* to microsomes does not necessarily provide results that are equivalent to those that occur following the *in vivo* administration of the drug to an intact animal. In light of

this discrepancy, and in the absence of a suitable *in vitro* method, cimetidine inhibition studies should be conducted with the drug administered *in vivo* in order to avoid making erroneous conclusions regarding the enzyme selectively or the mechanism of inhibition by the compound. However, there are limitations in performing inhibition studies with a putative inhibitor administered *in vivo* to an intact animal. Therefore, there is an obvious need to find a suitable *in vitro* method that will adequately model the inhibition that occurs following the *in vivo* administration of an inhibitor.

1.3 OBJECTIVES

The overall goal of this investigation was to provide a better understanding of the inhibition of hepatic cytochrome P-450 by *in vivo* and *in vitro* cimetidine treatment. The following were the objectives:

A. to determine whether cimetidine, when administered *in vivo* to adult male rats, differentially inhibits hepatic microsomal cytochrome P-450-mediated enzyme activities,

B. to determine whether the differential inhibition of cytochrome P-450-mediated enzyme activities by *in vivo* cimetidine, if present, occurs when cimetidine is added to hepatic microsomes *in vitro*, and

C. to determine the effect of preincubation on the inhibition of cytochrome P-450 enzyme activities by *in vitro* cimetidine treatment.

MATERIALS AND METHODS

2.1 CHEMICALS

Cimetidine hydrochloride was a gift from Smith Kline & French Canada Ltd. (Montreal, Que.). Aminopyrine, erythromycin base, dexamethasone, β-naphthoflavone, semicarbazide hydrochloride, ammonium acetate, acetylacetone, testosterone, androstenedione and 16-keto-testosterone were obtained from Sigma Chemical Company (St. Louis, MO). 6β-, 7α-, 11β-, 16α, and 16β-hydroxytesto-sterone were bought from Steraloids, Inc. (Wilton, NH). 2α- and 2β-hydroxytestosterone were provided by Professor D.N. Kirk, MRC Steroid Reference Collection, Queen Mary's College, London, United Kingdom. Ethoxyresorufin (7-ethoxyphenoxazone) and pentoxyresorufin (7-pentoxyphenoxazone) were supplied by Molecular Probes, Inc. (Eugene, OR). Resorufin (phenoxazone) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Phenobarbital sodium and NADPH were purchased from British Drug House (Toronto, Ont.) and Boehringer Mannheim Canada Ltd. (Dorval, Que.), respectively. Formaldehyde was obtained from Fisher Scientific Company (Fair Lawn, NJ). Bovine serum albumin was purchased as part of the Bio-Rad Protein Assay Kit[R] (Bio-Rad Laboratories, Mississauga, Ont.). Monospecific polyclonal rabbit-anti-rat cytochrome

P450IIC11 antibody was generously provided by Dr. S. M. Bandiera of the Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, British Columbia, Canada. Control rabbit IgG (ChromPure[R]) was supplied by Jackson Immunoresearch Lab. Inc. (West Grove, PA). All other chemicals were reagent grade.

2.2 ANIMALS

Adult male Wistar rats (51-55 days old, weighing 250-300 g) were obtained from Canadian Breeding Farms (Montreal, Que.) and were allowed to acclimatize in our animal care facility for at least seven days prior to initiation of treatment. The temperature of the animal room was maintained at 22°C and fluorescent lighting in the room was controlled by an automatic timer (0800 h on, 2200 h off). The animals were housed on Lobund[R] corncob bedding (Paxton Processing Ltd., Paxton Processing Ltd., Paxton, IL) and were provided with Rodent Laboratory Chow #5001[R] (Ralston Purina Canada Inc., Longueuil, Que.) and tap water *ad libitum* up to the time of sacrifice.

2.3 TREATMENT

Phenobarbital sodium and cimetidine hydrochloride were dissolved in distilled water. Dexamethasone was suspended in 2% w/v Tween 80 (e.g. Schuetz and Guzelian, 1984; Wrighton *et al.*, 1985a), whereas β-naphthoflavone was

suspended in corn oil (e.g. Guengerich *et al.*, 1982b; Thomas *et al.*, 1983; Waxman, 1984). All injections were by the intraperitoneal route.

2.3.1 Induction Protocol (Pretreatment)

Rats were pretreated with a compound known to preferentially induce the cytochrome P-450 enzyme(s) of interest. To induce cytochrome P450IIB1 and cytochrome P450IIB2, rats were pretreated with phenobarbital sodium, 80 mg/kg once daily for four days. Rats pretreated in this manner will be referred to as "phenobarbital-induced rats". Control rats received 0.9% saline. To induce cytochrome P450IIIA1, rats were pretreated with dexamethasone, 100 mg/kg once daily for 3 days. Rats pretreated in this manner will be referred to as "dexamethasone-induced rats". Control rats received the vehicle, 2% w/v Tween 80. To induce cytochrome P450IA1, rats were pretreated with β-naphthoflavone, 40 mg/kg once daily for three days. Rats pretreated in this manner will be referred to as "β-naphthoflavone-induced rats". Control rats received the vehicle, corn oil. These are standard injection protocols for maximally inducing the cytochrome P-450 enzymes indicated above (e.g. Guengerich *et al.*, 1982b; Waxman, 1984; Halpert *et al.*, 1985a; Dutton and Parkinson, 1989). The saline- or vehicle-treated rats (four rats per group) were included in each induction/inhibition study as an

internal control group. To study the cytochrome P-450 enzymes in the uninduced state, the rats were not pretreated (e.g. Guengerich *et al.*, 1982b; Waxman *et al.*, 1985; Wrighton *et al.*, 1985a). The term "uninduced rats" will be used to refer to those animals not subjected to any injections during the pretreatment phase.

2.3.2 Inhibition Protocol (Treatment)

In the *in vivo* inhibition experiments, a single dose of cimetidine hydrochloride (150 mg/kg) or 0.9% saline (uninhibited control) was administered to uninduced and induced rats 24 h after the last pretreatment dose. In cases where the drug was administered in this manner, the term "*in vivo* cimetidine" will be used. This dosage of cimetidine has been used by other investigators (Drew *et al.*, 1981; Reichen *et al.*, 1986). In a preliminary experiment, a dose of 150 mg/kg intraperitoneally yielded maximal inhibition of aminopyrine N-demethylase activity when rats were sacrificed 90 min after the cimetidine injection.

In the *in vitro* inhibition experiments, cimetidine hydrochloride, which was dissolved in distilled water, was added directly to the incubation mixture. In cases where the drug was added in this manner, the term "*in vitro* cimetidine" will be used.

2.4 TIME OF SACRIFICE

In the *in vivo* cimetidine experiments, rats were sacrificed 90 min after the single injection of cimetidine or saline. In the previous studies reported by other investigators, the time of sacrifice varied between one to two hours (Drew *et al.*, 1981, Mosca *et al.*, 1985; Yee and Shargel, 1986). In a preliminary experiment, maximal inhibition of aminopyrine N-demethylase activity was observed when rats were sacrificed 90 min after a single injection of cimetidine.

In the other experiments, the animals were sacrificed 24 h after the last dose of the inducer or vehicle (e.g. Gontovnick and Bellward, 1980).

2.5 PREPARATION OF HEPATIC MICROSOMES

Hepatic microsomes were prepared by a standard method (Lu and Levin, 1972). Each rat was stunned, decapitated and exsanguinated. Immediately after the abdominal cavity was opened, the liver was excised and immersed in 20 mL of 50 mM TRIS / 1.15% potassium chloride (pH 7.5) at 4 °C. All subsequent procedures were performed at 4 °C. Depending on the particular experiment, either individual or pooled livers were homogenized in the 50 mM TRIS / 1.15% potassium chloride buffer using a Potter-Elvejhem homogenizer with a motor-driven teflon pestle. The homogenate was centrifuged at 10,000 x g for 20 min. Following centrifugation, the

supernatant was filtered through four layers of cheesecloth to remove the surface lipid and was then subjected to ultracentrifugation at 100,000 x g for 60 min. The pellet was suspended in 20 mL of the 10 mM EDTA / 1.15% potassium chloride (pH. 7.4) buffer and recentrifuged at 100,000 x g for 60 min. The microsomal pellet was resuspended in 4 mL of 0.25 M sucrose. Aliquots (0.5 - 1 mL) of each microsomal preparation were placed in cryotubes and stored at -80 °C until use. When prepared by this method, the microsomes were found to be stable for at least two years (Thomas *et al.*, 1983).

2.6 DETERMINATION OF TOTAL CYTOCHROME P-450 CONTENT

Total microsomal cytochrome P-450 content was determined from the sodium dithionite-reduced carbon monoxide difference spectrum (Omura and Sato, 1964) using a molar extinction coefficient of 91 cm^{-1} mM^{-1} between 450 nm and 490 nm. Microsomal suspensions were diluted in a buffer containing 100 mM potassium phosphate (pH 7.4), 20% v/v glycerol and 0.1 mM EDTA (Thomas *et al.*, 1983). Microsomes from uninduced rats were diluted to 1:20 v/v, while those from induced rats were diluted to 1:50 v/v. A few milligrams of sodium dithionite were placed in both the sample and reference cuvettes containing the diluted microsomes. The contents of the sample cuvette were then gently saturated with carbon monoxide for 60 seconds at

approximately 1 bubble per second. After a few minutes the spectrum was recorded from 325 nm to 625 nm at room temperature. The scanning was repeated until no further increase in the absorbance at 450 nm was observed. All determinations were performed in duplicate using a SLM-Aminco DW-2 scanning spectrophotometer.

2.7 MICROSOMAL PROTEIN ASSAY

Microsomal protein concentration was determined by the method of Bradford (1976) with the Bio-Rad Protein Assay Kit[R]. Absorbance was measured at 595 nm using a Hewlett-Packard Model 8452A diode array spectrophotometer. The concentration of the unknown sample was determined from a standard curve of absorbance versus the concentration of bovine serum albumin. All determinations were performed in duplicate.

2.8 ENZYME ASSAYS

2.8.1 Enzyme Assay Conditions

Preliminary experiments were performed for each enzyme assay with microsomes from uninduced and induced rats to ensure that the amount of product formed was linear with respect to incubation time and microsomal protein concentration. The concentration of the substrate and the

concentration of NADPH used in each assay were shown to yield maximal product formation.

2.8.2 Aminopyrine N-Demethylase Assay

The N-demethylation of aminopyrine was estimated spectrophotometrically by a standard method (Gontovnick and Bellward, 1980) with modifications. The production of formaldehyde was measured by the method of Nash (1953). The reaction mixture, in a final volume of 1.5 mL, included: 1 mL of 100 mM potassium phosphate (pH 7.4); 0.1 mL of 62.5 mM semicarbazide hydrochloride; 0.1 mL of 111.5 mM magnesium chloride; 0.1 mL of 15 mM aminopyrine dissolved in distilled water; and 0.1 mL of microsomes diluted in 0.25 M sucrose. The final substrate concentration was 1 mM. The final protein concentrations in the reaction mixture were 0.5 mg/mL and 0.15 mg/mL for microsomes from uninduced and induced rats, respectively. After the mixture was preincubated for 75 seconds at 37 °C in a shaking water bath, the reaction was initiated by the addition of 0.1 mL of 15 mM NADPH (dissolved in 100 mM potassium phosphate, pH 7.4). The reaction was allowed to proceed for 10 min at 37 °C before being terminated by the addition of 0.5 mL of ice-cold 20% w/v trichloroacetic acid. After mixing on a vortex, each test tube was placed on ice. Substrate and microsomes were then added to the blank tubes that were preincubated without the substrate and microsomes,

respectively. All tubes were centrifuged for 15 min at 1,000 x g. The supernatant (1.5 mL) was transferred to a new test tube followed by the addition of 0.5 mL of Nash reagent (15 g of ammonium acetate, 0.2 mL of acetylacetone in 50 mL of distilled water). The tubes were incubated at 60 °C for 15 min in a shaking water bath. After the samples were allowed to cool to room temperature under the fume hood, the intensity of the yellow colour produced was measured on a Hewlett-Packard Model 8452A diode array spectrophotometer. The amount of formaldehyde produced was determined from a standard curve of absorbance at 412 nm versus formaldehyde concentration. The standard samples contained inactivated microsomes (inactivated in a 60 °C water bath). All determinations were performed in duplicate. The mean absorbance of the blank samples was subtracted from the mean absorbance of the experimental samples. Aminopyrine N-demethylase activity is expressed as nanomoles of formaldehyde formed per min per mg of microsomal protein.

2.8.3 Pentoxyresorufin O-Dealkylase Assay

The O-dealkylation of pentoxyresorufin was determined fluorometrically by the formation of resorufin (Lubet *et al.*, 1985). The reaction mixture, in a final volume of 2 mL, included: 1.93 mL of 100 mM HEPES / 5 mM magnesium chloride (pH 7.8), 10 µL of 1 mM pentoxyresorufin dissolved

in dimethylsulfoxide and 50 μL of microsomes diluted in 0.25 M sucrose. The final substrate concentration was 5 μM. The final protein concentrations in the reaction mixture were 150 μg/mL and 50 μg/mL for microsomes from uninduced and induced rats, respectively. The reaction was carried out in an optical glass fluorescence cell (1 cm path length) at 37 °C and initiated by the addition of 10 μL of 50 mM NADPH (dissolved in 100 mM HEPES / 5 mM magnesium chloride, pH 7.8). The fluorescence reading was recorded after a 5 min reaction period. The quantity of resorufin formed was determined from a standard curve of fluorescence versus resorufin concentration. The standard samples contained inactivated microsomes. The fluorescence of the blank sample (without NADPH) was subtracted from the experimental sample. Both pentoxyresorufin and resorufin were dissolved in dimethylsulfoxide (Burke *et al.* 1985) and stored in the dark. The experimental procedures were performed under subdued lighting with the overhead light off. All determinations were performed in duplicate using a Shimadzu RF-540 spectrophotofluorometer interfaced with a Shimadzu DR-3 data recorder. The excitation wavelength was set at 530 nm (slit width, 5 nm) and the emission wavelength was set at 582 nm (slit width, 5 nm). Pentoxyresorufin O-dealkylase activity is expressed as nanomoles of resorufin formed per min per mg of microsomal protein.

2.8.4 Erythromycin N-Demethylase Assay

The N-demethylation of erythromycin was estimated spectrophotometrically (Arlotto *et al.*, 1987). The production of formaldehyde was measured by the method of Nash (1953). The reaction mixture, in a final volume of 1.5 mL, included: 1 mL of 100 mM potassium phosphate (pH 7.4); 0.1 mL of 75 mM semicarbazide hydrochloride; 0.1 mL of 45 mM magnesium chloride; 0.1 mL of 6 mM erythromycin base dissolved in 30% v/v ethanol; and 0.1 mL of microsomes diluted in 0.25 M sucrose. The final substrate concentration was 0.4 mM. The final protein concentrations in the reaction mixture were 0.6 mg/mL and 0.15 mg/mL for microsomes from uninduced and induced rats, respectively. The other steps were the same as those described for the aminopyrine N-demethylase assay. Erythromycin N-demethylase activity is expressed as nanomoles of formaldehyde formed per min per mg of microsomal protein.

2.8.5 Ethoxyresorufin O-Deethylase Assay

The O-deethylation of ethoxyresorufin was determined fluorometrically by resorufin formation (Burke and Mayer, 1974). This assay was performed using the same experimental procedures and conditions as the pentoxyresorufin O-dealkylase assay except that ethoxyresorufin was the substrate. Ethoxyresorufin O-deethylase activity is

expressed as nanomoles of resorufin formed per min per mg of microsomal protein.

2.8.6 Testosterone Oxidase Assay

The microsomal hydroxylation and oxidation of testosterone was determined by the method of Wood *et al.* (1983). The reaction mixture, in a final volume of 1 mL, included: 0.5 mL of 100 mM potassium phosphate (pH 7.4); 0.1 mL of 30 mM magnesium chloride; 0.2 mL of 0.25 M sucrose; 20 μL of 12.5 mM testosterone dissolved in methanol; and 80 μL of microsomes diluted in 0.25 M sucrose. The final substrate concentration was 0.25 mM. The final protein concentrations in the reaction mixture were 0.5 mg/mL and 0.15 mg/mL for samples from uninduced and induced rats, respectively. After the mixture was preincubated for 75 seconds at 37 °C in a shaking water bath, the reaction was initiated by adding 0.1 mL of 10 mM NADPH dissolved in 50 mM potassium phosphate (pH 7.4) to each incubation tube. The reaction was allowed to proceed for 5 min at 37 °C before it was terminated by the addition of 6 mL of methylene chloride. Subsequently, 0.1 mL of internal standard (16-keto-testosterone or 11β-hydroxytestosterone, 3 nmol per tube) was added and the incubation contents were mixed on a vortex for 30 seconds. The samples were centrifuged for 2 min at 800 x g. The aqueous phase (upper layer) was aspirated and 4 mL of the organic phase was transferred to a

test tube and evaporated under a stream of nitrogen at 35 °C. The residue was dissolved in 0.2 mL of methanol. The sample was filtered through a 0.45 μm Type HV filter (Millipore Ltd., Mississauga, Ont.). A volume of 10 μL was used for high performance liquid chromatographic analysis.

Formation of microsomal testosterone oxidation products was quantitated by a high performance liquid chromatographic method based on that described by Wood *et al.* (1983). The system consisted of two Waters Model 501 pumps, an automatic sample injector (Waters Model 712 WISP[R]) and a Waters Model 484 ultraviolet-visible absorbance detector. A software program, BASELINE 810 Chromatography Workstation[R], was used to control the operation of these devices as well as for data analysis.

All samples were analyzed using a reverse phase column preceded by a Pelliguard[R] LC-18, 2 cm guard column (Supelco, Inc., Bellefonte, PA). In samples from uninduced and dexamethasone-treated rats, the separation of 2α-, 2β-, 16α- and 16β-hydroxytestosterone as well as androstenedione was performed using a 5 μm octyldecylsilane, 4.6 x 150 mm inner diameter, reverse phase column (Supelco, Inc., Bellefonte, PA). A concave gradient (Option No.7 in the BASELINE 810 software program) from 100% Solvent A to 100% Solvent B was used over a 25 min period at a flow rate of 1.5 mL/min. Solvent A contained 465 mL of methanol, 530 mL of distilled water and 11 mL of acetonitrile. Solvent B contained 760 mL

of methanol, 220 mL of distilled water and 11 mL of acetonitrile. The same conditions were used for the separation of these metabolites in samples from phenobarbital-induced rats except that the composition of Solvent A was 430 mL of methanol, 600 mL of distilled water and 11 mL of acetonitrile and the gradient time was 33 min. Methanol and acetonitrile were HPLC grade. Water was distilled and further purified with a Millipore Milli-Q apparatus (Millipore Ltd., Mississauga, Ont.).

For the separation of 6β- and 7α-hydroxytestosterone in microsomes from all groups, different chromatographic conditions were used to improve the resolution of these two metabolites. The main column was a Zorbax[R] 5 μm octyldecylsilane, 4.6 x 150 mm diameter, reverse phase column (Dupont Canada Inc., Mississauga, Ont.). The mobile phase was 14% tetrahydrofuran and 86% distilled water for the first 18 min followed by a mobile phase of 69% tetrahydrofuran and 31% distilled water for an additional 5 min. The flow rate was 1.5 mL/min.

An unidentified enzymatic product co-eluted with 16-keto-hydroxytestosterone in samples from phenobarbital-induced rats. Consequently, the internal standard used in these samples was 11β-hydroxytestosterone. In all other cases, 16-keto-testosterone was used as the internal standard.

All chromatographic separations were performed at room temperature. The absorbance of the column effluents was monitored at 254 nm. Metabolites were identified by comparing retention times to those of authentic standards. The amount of each metabolite formed was determined by linear regression analysis of a standard curve of peak height ratio (analyte/internal standard) versus concentration ratio (analyte/internal standard). Peak height was calculated by the BASELINE 810 software program. Inactivated microsomes were used in the standard samples. All determinations were performed in duplicate. Activity is expressed as nanomoles of testosterone metabolite formed per min per mg of microsomal protein.

2.9 IMMUNOINHIBITION STUDIES

The effect of a preparation of monospecific polyclonal anti-cytochrome P450IIC11 antibody on aminopyrine N-demethylase, pentoxyresorufin 0-dealkylase, erythromycin N-demethylase and testosterone oxidase activities was determined by a standard method (Thomas *et al.*, 1981). Hepatic microsomes (0.3 nmol of total cytochrome P-450) from uninduced, phenobarbital- or dexamethasone-induced rats were preincubated with anti-cytochrome P450IIC11 antibody or control rabbit IgG in phosphate-buffered saline (pH 7.4) for 10 min at room temperature. (Phosphate-buffered saline contained 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4

and 0.2 mM EDTA). Each test tube contained 0.25, 0.5, 1, 2.5, 5 or 10 mg of anti-cytochrome P450IIC11 antibody or control rabbit IgG per nmol of total cytochrome P-450. Substrate oxidation was initiated as described above (see Sections 2.8.2, 2.8.3, 2.84 and 2.86). Microsomes prepared from pooled livers were used. All determinations were performed in duplicate. Results are expressed as a percent of control activity.

2.10 STATISTICAL ANALYSES

In experiments where microsomal samples isolated from individual livers were used, the data were subjected to formal statistical analysis using the UBC SPSS-X computer program (Lai, 1986). The significance of the difference between the means of two treatment groups was evaluated by the two-tailed independent Student's t-test. The a priori level of significance was set at $p < 0.05$. Statistical analyses were not performed on the data obtained from experiments where the microsomal samples were prepared from pooled livers. In these cases, the sample size was unity.

RESULTS

3.1 STUDIES WITH *IN VIVO* CIMETIDINE

To determine whether hepatic cytochrome P-450 enzymes are inhibited selectively by *in vivo* cimetidine treatment in adult male rats, the animals were pretreated as described in "Materials and Methods" (Section 2.3.1). The treatment phase for studying inhibition consisted of a single intraperitoneal injection of cimetidine (150 mg/kg) or saline (uninhibited control) as described in "Materials and Methods" (Section 2.3.2). In cases where the purpose was to examine the effect of cimetidine on a particular cytochrome P-450 in microsomes, an enzyme-specific activity was determined. It is important to note that in the different enzyme assays, aliquots of the *same* microsomal suspension were used.

3.1.1 Total Cytochrome P-450 Content

Phenobarbital, dexamethasone and β-naphthoflavone increased the total microsomal cytochrome P-450 content by 1.9-, 1.5- and 1.5-fold, respectively (Table 6). *In vivo* cimetidine treatment did not change the total cytochrome P-450 content in microsomes from uninduced, phenobarbital-, dexamethasone- or β-naphthoflavone-induced rats (Figure 3).

TABLE 6

EFFECT OF PHENOBARBITAL, DEXAMETHASONE AND β-NAPHTHOFLAVONE
ON TOTAL MICROSOMAL CYTOCHROME P-450 CONTENT

Pretreatment	N	Total Cytochrome P-450 (nmol/mg protein)
Saline	4	1.54 ± 0.05
Phenobarbital	8	2.98 ± 0.27*
2% Tween 80	4	1.51 ± 0.08
Dexamethasone	8	2.29 ± 0.07*
Corn Oil	4	1.14 ± 0.07
β-naphthoflavone	8	1.73 ± 0.09*

Results are expressed as the mean ± SEM for the number
(N) of rats indicated. *$p < 0.05$, compared to the
corresponding control group.

Figure 3 Effect of *in vivo* cimetidine on total microsomal cytochrome P-450 content. Results are expressed as the mean ± SEM for 8 rats per group.
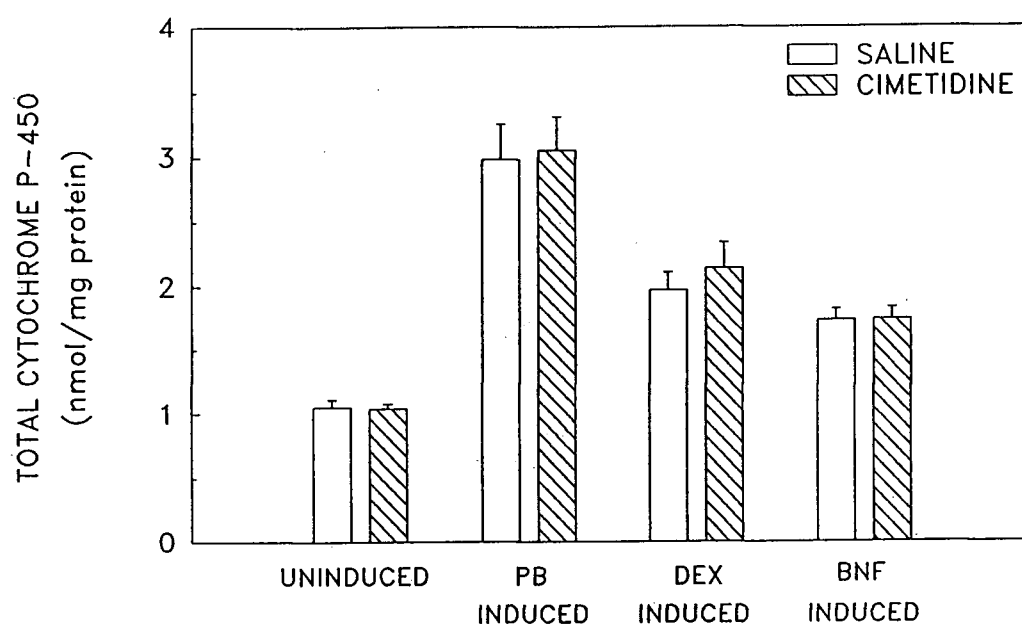
3.1.2 Aminopyrine N-Demethylase Activity

Aminopyrine is a substrate which is known to be demethylated by many non-inducible and inducible cytochrome P-450 enzymes (Guengerich *et al.*, 1982a). Since the inducing agents used in this study were known to induce different cytochrome P-450 enzymes, aminopyrine N-demethylase was used initially as a non-selective marker to probe for differential inhibition by cimetidine.

Phenobarbital and dexamethasone pretreatment increased aminopyrine N-demethylase activity by 2.6- and 2.2-fold, respectively, whereas β-naphthoflavone pretreatment decreased this activity by 0.46-fold (Table 7). *In vivo* cimetidine inhibited aminopyrine N-demethylase activity by 62% in microsomes from uninduced rats, whereas it inhibited this activity by only 33%, 20% and 28% in microsomes from rats induced with phenobarbital, dexamethasone and β-naphthoflavone, respectively (Figure 4). The apparent increased inhibition of aminopyrine N-demethylase activity by *in vivo* cimetidine in the uninduced rats suggested that at least one of the cytochrome P-450 enzymes present in these animals is more susceptible to inhibition by cimetidine than are the inducible cytochrome P-450 enzymes.

Based on this observation, subsequent experiments were designed to study the effects of cimetidine on substrates known to be specifically metabolized by particular

TABLE 7

EFFECT OF PHENOBARBITAL, DEXAMETHASONE AND β-NAPHTHOFLAVONE
ON AMINOPYRINE N-DEMETHYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	5.84 ± 0.61
Phenobarbital	8	15.20 ± 0.84*
2% Tween 80	4	6.34 ± 0.68
Dexamethasone	8	13.96 ± 0.70*
Corn Oil	4	6.36 ± 0.74
β-naphthoflavone	8	2.90 ± 0.26*

Results are expressed as the mean ± SEM for the number
(N) of rats indicated. *p < 0.001, compared to the
corresponding control group.

Figure 4   Effect of *in vivo* cimetidine on aminopyrine N-demethylase activity.   Results are expressed as the mean ± SEM for 8 rats per group.   *p < 0.02, #p < 0.001, compared to the corresponding saline-treated group.
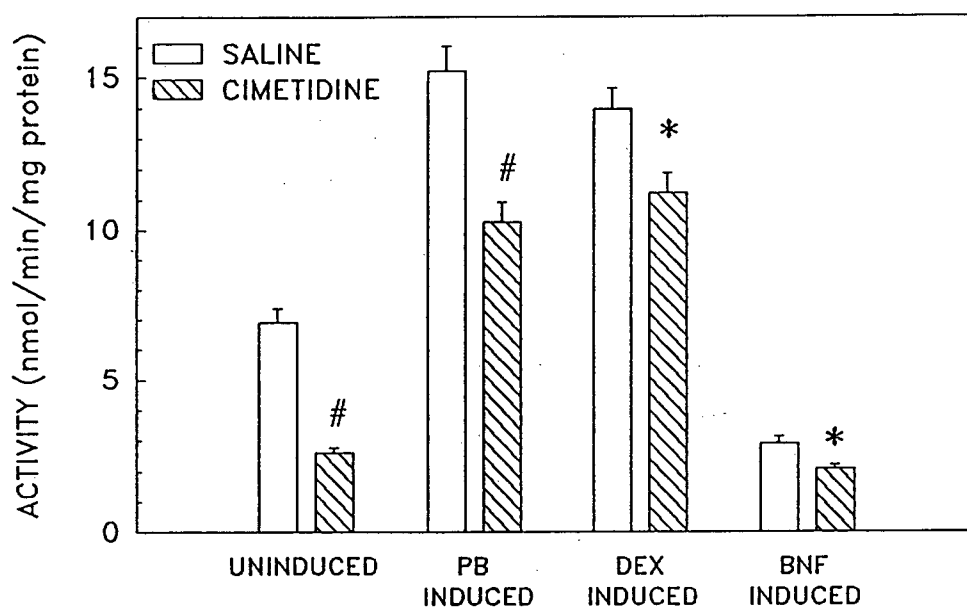
cytochrome P-450 enzymes under defined pretreatment conditions.

3.1.3 Pentoxyresorufin O-Dealkylase Activity

In vivo cimetidine inhibited pentoxyresorufin O-dealkylase activity by 38% in microsomes from uninduced rats (Figure 5A). Phenobarbital and dexamethasone pretreatment increased pentoxyresorufin O-dealkylase activity by 108- and 13-fold, respectively (Table 8). *In vivo* cimetidine had no effect on this activity in microsomes from phenobarbital- or dexamethasone-induced rats (Figures 5A and 5B).

Cytochromes P450IIB1/2 account for more than 90% of the pentoxyresorufin O-dealkylase activity in hepatic microsomes from phenobarbital-induced rats (Lubet *et al.*, 1985; Waxman *et al.*, 1987; Dutton and Parkinson, 1989). Hepatic microsomes from uninduced rats contain low levels of cytochromes P450IIB1/2 (Guengerich *et al.*, 1982a; Thomas *et al.*, 1983; Waxman *et al.*, 1985). The major cytochrome P-450 enzymes responsible for pentoxyresorufin O-dealkylase activity in microsomes from uninduced rats have not been identified, but it has been reported that cytochrome P450IIB1/2 do not contribute to this activity in these microsomes (Waxman *et al.*, 1987). Although dexamethasone pretreatment induces cytochrome P450IIB1/2 (Yamazoe *et al.*, 1987), it has not yet been determined which cytochrome P-450

Figure 5 Effect of *in vivo* cimetidine on pentoxyresorufin 0-dealkylase activity. Microsomes were isolated from (A) uninduced, (B) phenobarbital-induced and (C) dexamethasone-induced rats. Results are expressed as the mean ± SEM for 8 rats per group. *p < 0.02, compared to the corresponding saline-treated group.
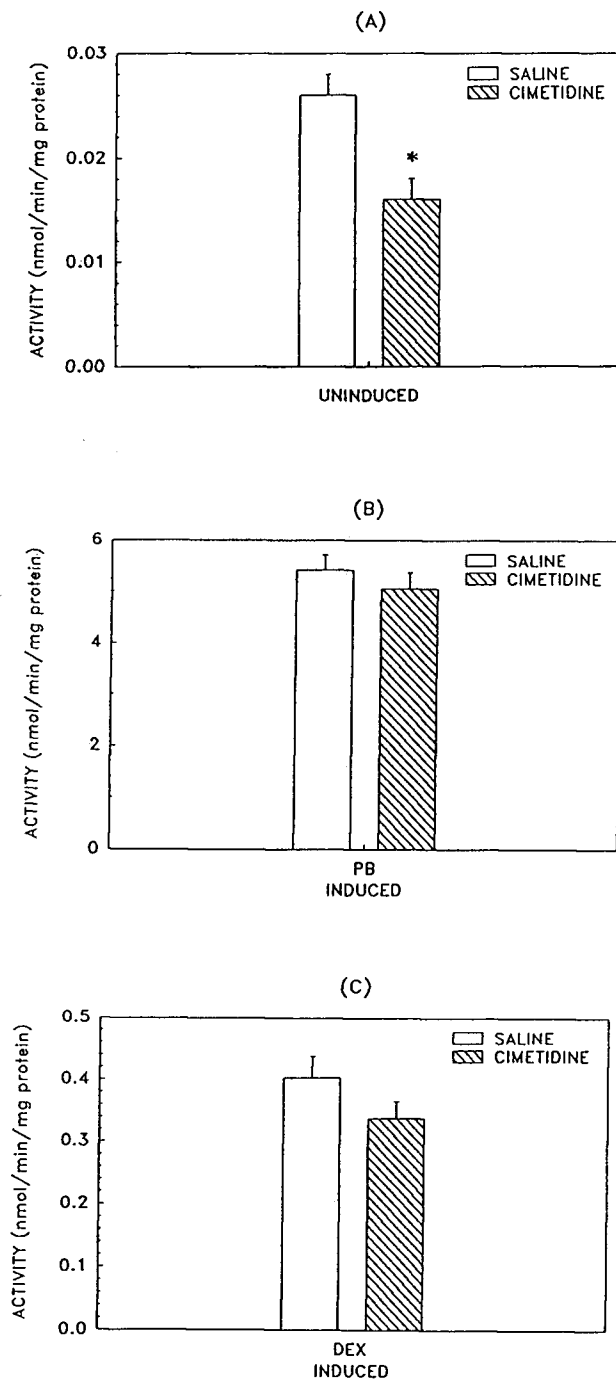
TABLE 8

EFFECT OF PHENOBARBITAL AND DEXAMETHASONE
ON PENTOXYRESORUFIN O-DEALKYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	0.05 ± 0.01
Phenobarbital	8	$5.42 \pm 0.30^*$
2% Tween 80	4	0.03 ± 0.01
Dexamethasone	8	$0.40 \pm 0.03^*$

Results are expressed as the mean $\pm$ SEM for the number (N) of rats indicated. $^*p < 0.001$, compared to the corresponding control group.

enzymes contribute to pentoxyresorufin 0-dealkylase activity following dexamethasone pretreatment.

3.1.4 Erythromycin N-Demethylase Activity

In vivo cimetidine inhibited erythromycin N-demethylase activity by 40% in microsomes from uninduced rats (Figure 6A). Phenobarbital and dexamethasone pretreatment increased this activity by 2.8- and 9.8-fold, respectively (Table 9). _In vivo_ cimetidine did not affect erythromycin N-demethylase activity in microsomes from either phenobarbital- or dexamethasone-induced rats (Figures 6B and 6C).

Cytochromes P450IIIA1/2 account for a majority of the erythromycin N-demethylase activity in hepatic microsomes from dexamethasone-induced rats (Wrighton _et al._, 1985a). Both cytochrome P450IIIA1 mRNA and cytochrome P450IIIA2 mRNA are present in livers of adult male rats treated with phenobarbital (Gonzalez _et al._, 1986), whereas cytochrome P450IIIA2, but not cytochrome P450IIIA1, is expressed in livers of uninduced adult male rats (Cooper _et al._, 1990). It has not yet been reported which cytochrome P-450 enzymes contribute to erythromycin N-demethylase activity in these microsomes from uninduced or phenobarbital-induced rats.

Figure 6 Effect of *in vivo* cimetidine on erythromycin N-demethylase activity. Microsomes were isolated from (A) uninduced, (B) phenobarbital-induced and (C) dexamethasone-induced rats. Results are expressed as the mean ± SEM for 8 rats per group. *p < 0.005, compared to the corresponding saline-treated group.

(A)

(B)

(C)
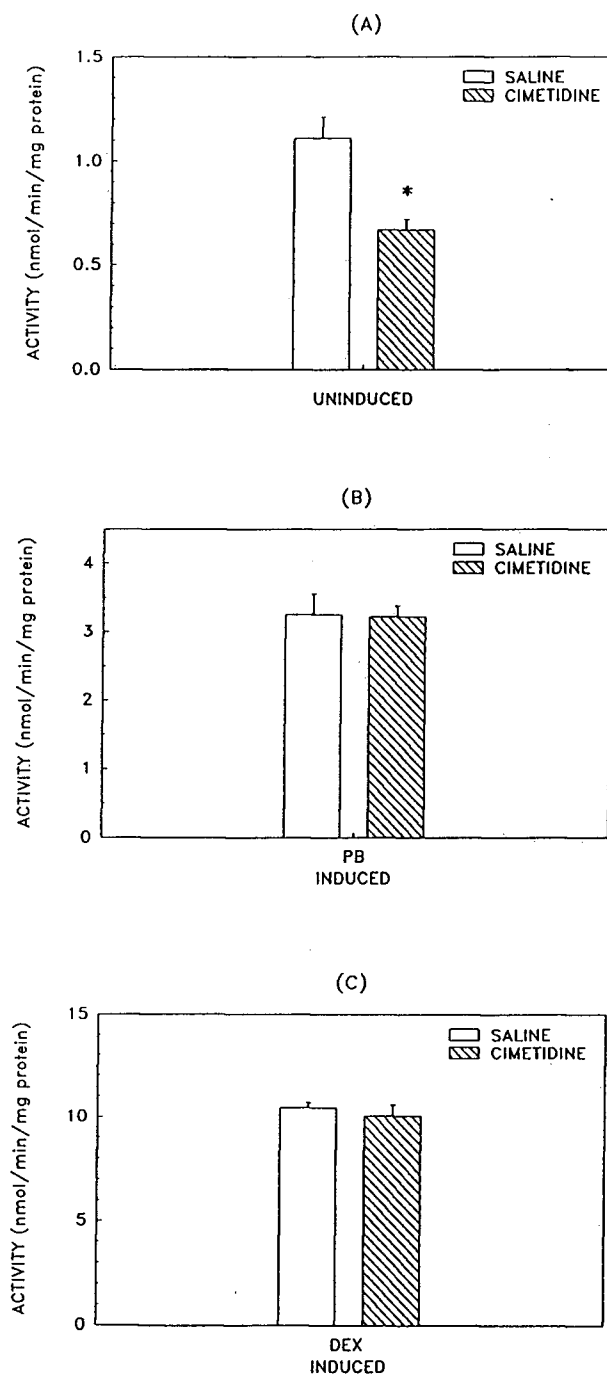
TABLE 9

EFFECT OF PHENOBARBITAL AND DEXAMETHASONE
ON ERYTHROMYCIN N-DEMETHYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	1.15 ± 0.12
Phenobarbital	8	$3.25 \pm 0.31^*$
2% Tween 80	4	1.06 ± 0.12
Dexamethasone	8	$10.44 \pm 0.27^*$

Results are expressed as the mean $\pm$ SEM for the number (N) of rats indicated. $^*p < 0.001$, compared to the corresponding control group.

3.1.5 Ethoxyresorufin O-Deethylase Activity

In vivo cimetidine inhibited ethoxyresorufin O-deethylase activity by 84% in microsomes from uninduced rats (Figure 7A). β-Naphthoflavone pretreatment increased this activity by 23-fold (Table 10). *In vivo* cimetidine did not affect ethoxyresorufin O-deethylase activity in microsomes from β-naphthoflavone-induced rats (Figure 7B).

It has been reported that, in hepatic microsomes from uninduced rats, cytochrome P450IA2 accounts for approximately 80% of the ethoxyresorufin O-deethylase activity and that cytochrome P450IA1 accounts for the remainder of the activity (Kelley *et al.*, 1987). Recently, Nakajima *et al.* (1990) showed that cytochrome P450IIC11 also contributed to this enzyme activity. Therefore, ethoxyresorufin O-deethylase activity in hepatic microsomes from uninduced rats may not be a specific marker for cytochrome P450IA2. Although β-naphthoflavone pretreatment induces both cytochrome P450IA1 and cytochrome P450IA2 (Guengerich *et al.*, 1982a; Waxman *et al.*, 1985), it has yet to be reported whether these enzymes contribute to ethoxyresorufin O-deethylase activity in microsome from β-naphthoflavone-induced rats.

Figure 7 Effect of *in vivo* cimetidine on ethoxyresorufin 0-deethylase activity. Microsomes were isolated from (A) uninduced and (B) β-naphthoflavone-induced rats. Results are expressed as the mean ± SEM for 8 rats per group. *p < 0.02, compared to the corresponding saline-treated group.

(A)

(B)
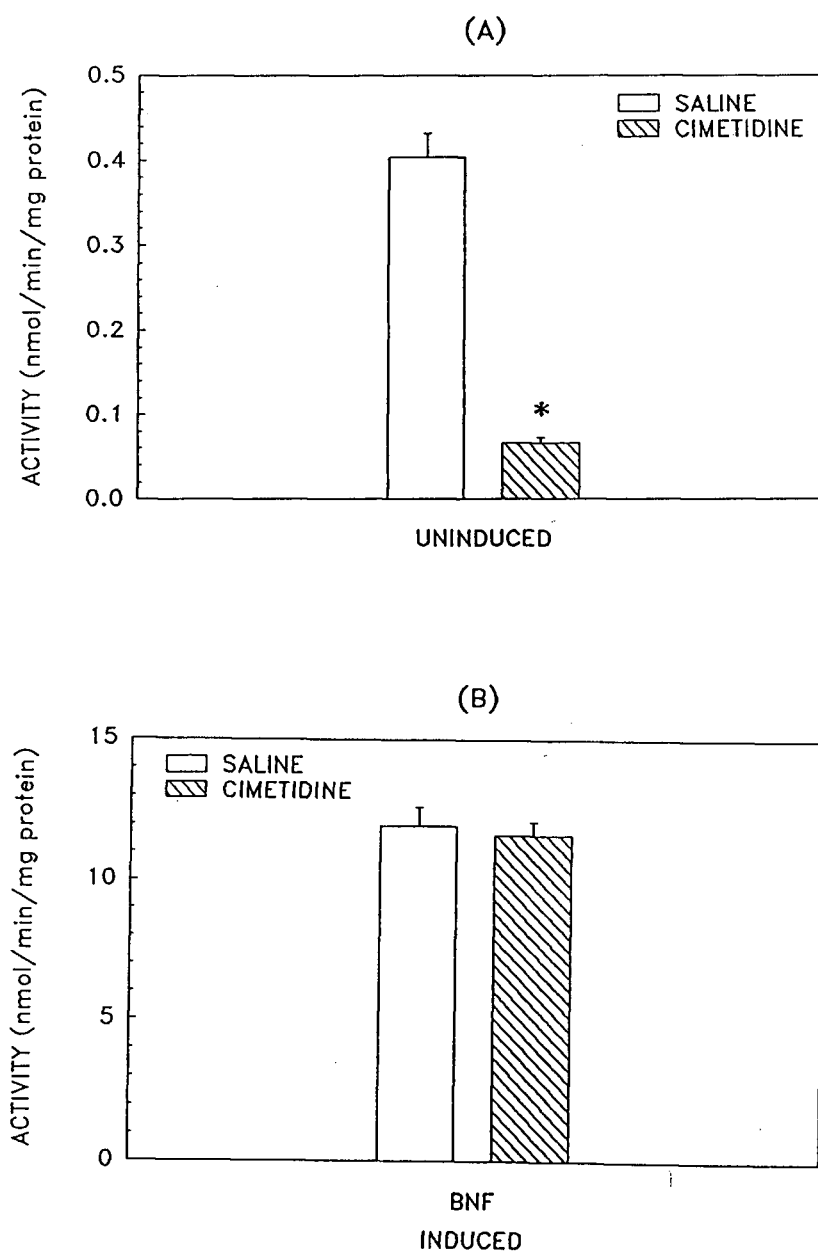
TABLE 10

EFFECT OF β-NAPHTHOFLAVONE
ON ETHOXYRESORUFIN O-DEETHYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	0.52 ± 0.06
β-Naphthoflavone	8	11.95 ± 0.65*

Results are expressed as the mean ± SEM for the number (N) of rats indicated. *p < 0.001, compared to the corresponding control group.

3.1.6 Testosterone Oxidase Activities

The results from the preceding sections indicated that several cytochrome P-450 enzyme activities (aminopyrine N-demethylase, pentoxyresorufin 0-dealkylase, erythromycin N-demethylase and ethoxyresorufin 0-deethylase) in microsomes from uninduced adult male rats were inhibited by *in vivo* cimetidine (Figures 8 and 9). In addition, some of these enzyme activities known to be specific for particular cytochrome P-450 enzymes in induced rats were not affected by cimetidine, indicating that these enzymes may not be subject to inhibition by this drug.

To further explore this apparent differential inhibition of cytochrome P-450 by cimetidine, microsomal testosterone oxidation was determined under defined pretreatment conditions. This was done because of the known specificity of several of the testosterone hydroxylase activities for particular cytochrome P-450 enzymes. For example, testosterone 2α- and 16α-hydroxylase activities in hepatic microsomes from uninduced adult male rats are specific for cytochrome P450IIC11 (Waxman, 1984; Waxman *et al.*, 1987). Since cytochrome P450IIC11 was known to be a major enzyme in livers of uninduced adult male rats (Guengerich *et al.*, 1982a; Dannan *et al.*, 1983; Waxman *et al.*, 1985)., this enzyme was a candidate for inhibition by cimetidine in these animals.

Figure 8   Summary of the effects of *in vivo* cimetidine on aminopyrine N-demethylase, pentoxyresorufin 0-dealkylase and erythromycin N-demethylase activities in microsomes from uninduced, phenobarbital-induced and dexamethasone-induced rats. Results are based on the data from Figures 4-6. #p < 0.001, &p < 0.005, *p < 0.02 compared to the saline-treated control group.
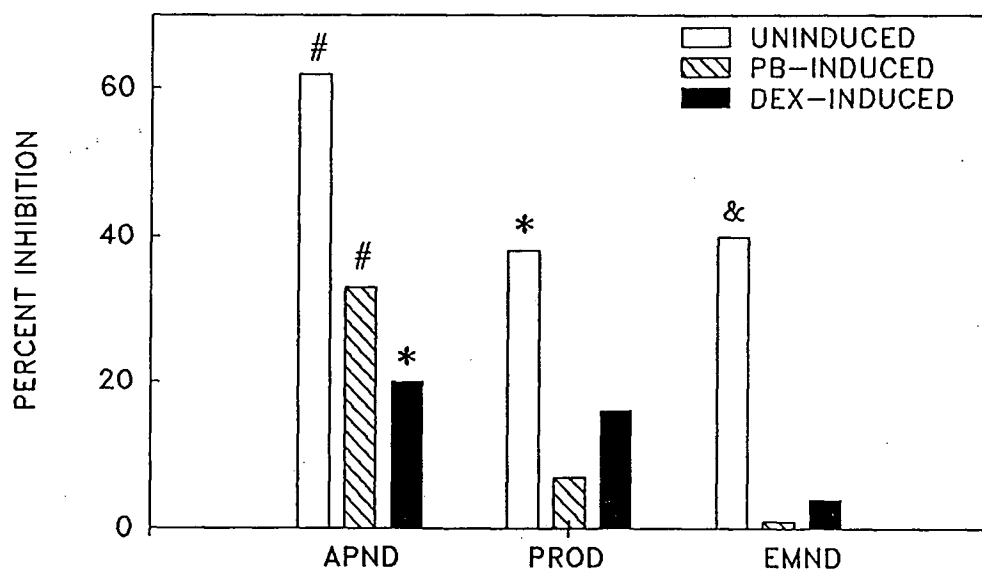
Figure 9 Summary of the effects of *in vivo* cimetidine on aminopyrine N-demethylase and ethoxyresorufin 0-deethylase activities in microsomes from uninduced and β-naphthoflavone-induced rats. Results are based on the data from Figure 4 and Figure 7. #p < 0.001, *p < 0.02 compared to the saline-treated control group.
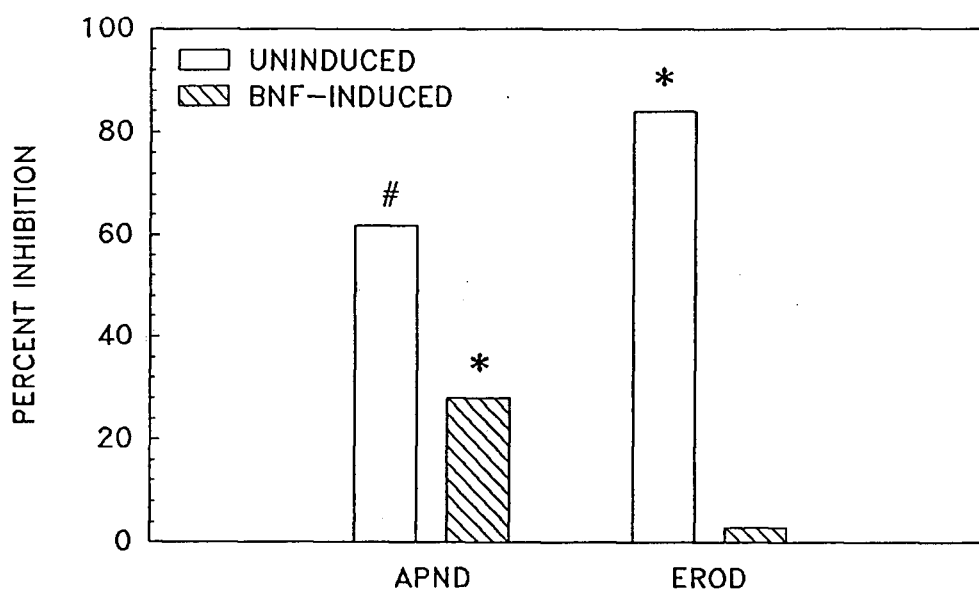
3.1.6.1   Testosterone 2α-Hydroxylase Activity

In vivo cimetidine inhibited testosterone 2α-hydroxylase activity by 65% in microsomes from uninduced rats (Figure 10).   It has been shown that cytochrome P450IIC11 accounts for more than 85% of this activity in hepatic microsomes from uninduced adult male rats (Waxman, 1984; Waxman *et al.*, 1987).

Phenobarbital pretreatment decreased testosterone 2α-hydroxylase activity by 51% (Table 11).   *In vivo* cimetidine inhibited the remaining activity by 73% in microsomes from phenobarbital-induced rats (Figure 10).   It is not known whether cytochrome P450IIC11 is the major enzyme responsible for this activity in microsomes from phenobarbital-induced adult male rats.

Dexamethasone pretreatment decreased testosterone 2α-hydroxylase activity by 58% (Table 11).   *In vivo* cimetidine inhibited 46% of the remaining activity in microsomes from dexamethasone-induced rats (Figure 10).   It is not known whether cytochrome P450IIC11 is the major enzyme responsible for this activity in microsomes from dexamethasone-induced adult male rats.

Figure 10 Effect of *in vivo* cimetidine on testosterone 2α-hydroxylase activity. Microsomes were isolated from uninduced, phenobarbital-induced and dexamethasone-induced rats. Results are expressed as the mean ± SEM. The sample size (N) was 8 rats per group, except for the uninduced, cimetidine-treated group (N = 7) and the dexamethasone-induced, cimetidine-treated group (N = 5). *p < 0.001, compared to the corresponding saline-treated control group.

TABLE 11

EFFECT OF PHENOBARBITAL AND DEXAMETHASONE
ON TESTOSTERONE 2α-HYDROXYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	2.38 ± 0.32
Phenobarbital	8	1.17 ± 0.13*
2% Tween 80	4	1.93 ± 0.34
Dexamethasone	8	0.81 ± 0.11*

Results are expressed as the mean ± SEM for the number (N) of rats indicated. *$p < 0.001$, compared to the corresponding control group.

3.1.6.2 Testosterone 2β- and 6β-Hydroxylase Activities

Phenobarbital pretreatment increased testosterone 2β- and 6β-hydroxylase activities by 6.1- and 3.7-fold, respectively (Tables 12 and 13). Dexamethasone pretreatment also increased these two activities, and the magnitude of the increase was 12-fold for testosterone 2β-hydroxylase activity and 5.5-fold for testosterone 6β-hydroxylase activity (Tables 12 and 13). *In vivo* cimetidine did not inhibit either testosterone 2β- or 6β-hydroxylase activity in microsomes from uninduced, phenobarbital- or dexamethasone-induced rats (Figures 11 and 12).

It has been reported that cytochromes P450IIIA1/2 account for more that 85% of the testosterone 2β- or 6β-hydroxylase activity in hepatic microsomes from uninduced, phenobarbital- or dexamethasone-induced adult male rats (Halvorson *et al.*, 1990). The results from the cimetidine experiments on testosterone 2β- and 6β-hydroxylase activities are consistent with the lack of inhibition of erythromycin N-demethylase activity in microsomes from dexamethasone-induced rats (Figure 6) since, in the latter case, cytochromes P450IIIA1/2 are also the major contributors (Wrighton *et al.*, 1985a).

TABLE 12

EFFECT OF PHENOBARBITAL AND DEXAMETHASONE ON
TESTOSTERONE 2β-HYDROXYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	0.07 ± 0.01
Phenobarbital	8	$0.43 \pm 0.03^*$
2% Tween 80	4	0.07 ± 0.02
Dexamethasone	8	$0.84 \pm 0.05^*$

Results are expressed as the mean ± SEM for the number
(N) of rats indicated. *$p < 0.001$, compared to the
corresponding control group.

TABLE 13

EFFECT OF PHENOBARBITAL AND DEXAMETHASONE ON
TESTOSTERONE 6β-HYDROXYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	2.36 ± 0.19
Phenobarbital	8	8.77 ± 0.88[*]
2% Tween 80	4	2.46 ± 0.37
Dexamethasone	8	13.53 ± 0.81[#]

Results are expressed as the mean ± SEM for the number (N) of rats indicated. [*]$p < 0.002$, compared to the corresponding control group. [#]$p < 0.001$, compared to the corresponding control group.

Figure 11 Effect of *in vivo* cimetidine on testosterone
2β-hydroxylase activity. Microsomes were isolated
from uninduced, phenobarbital-induced and
dexamethasone-induced rats. Results are expressed
as the mean ± SEM. The sample size (N) was 8 rats
per group, except for the uninduced, cimetidine-
treated group (N = 7) and the dexamethasone-
induced, cimetidine-treated group (N = 5).
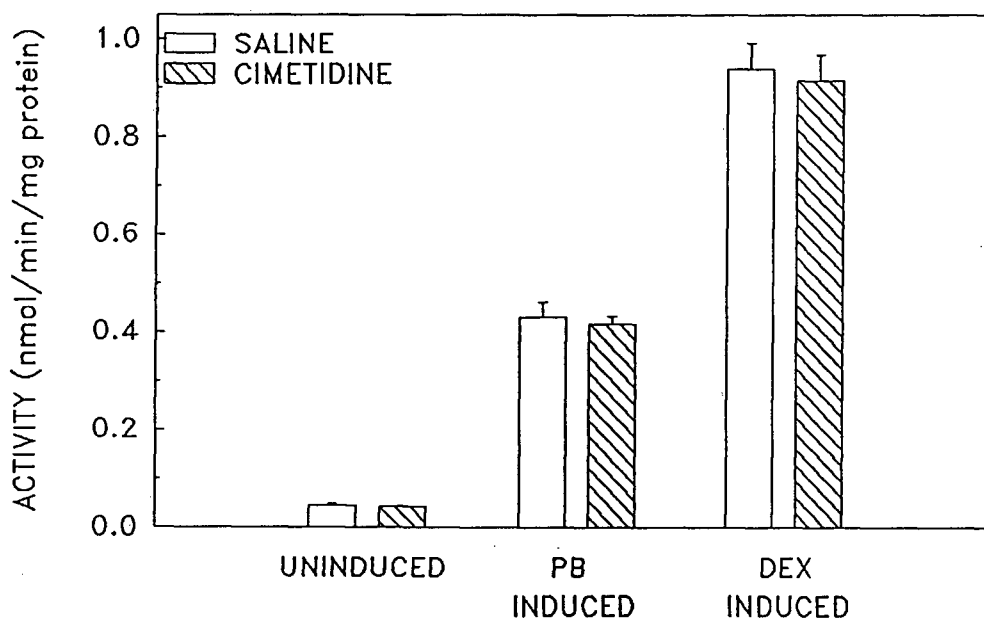
Figure 12 Effect of *in vivo* cimetidine on testosterone 6β-hydroxylase activity. Microsomes were isolated from uninduced, phenobarbital-induced and dexamethasone-induced rats. Results are expressed as the mean ± SEM. The sample size (N) was 8 rats per group, except for the uninduced, cimetidine-treated group (N = 7) and the dexamethasone-induced, cimetidine-treated group (N = 5).
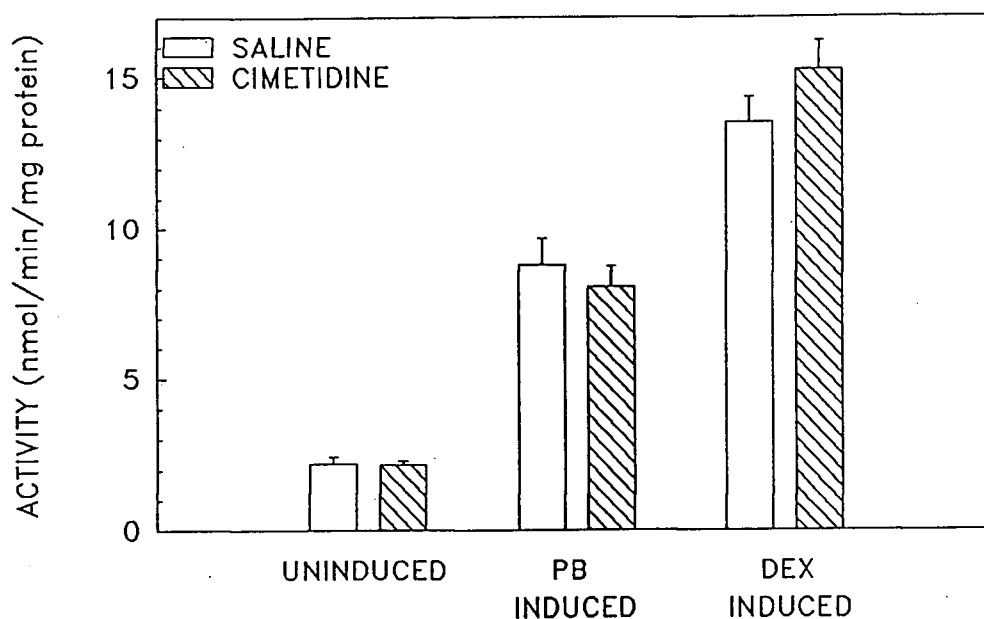
3.1.6.3   Testosterone 7α-Hydroxylase Activity

In vivo cimetidine did not inhibit testosterone 7α-hydroxylase activity in microsomes from uninduced rats (Figure 13). Neither phenobarbital nor dexamethasone had any effect on testosterone 7α-hydroxylase activity (Table 14). In vivo cimetidine did not inhibit this activity in microsomes from phenobarbital- or dexamethasone-induced rats (Figure 13).

It has been shown that cytochrome P450IIA1 accounts for 80-96% of the testosterone 7α-hydroxylase activity in hepatic microsomes from uninduced, phenobarbital- or dexamethasone-induced rats (Levin et al., 1987; Waxman et al., 1988b; Arlotto and Parkinson, 1989).

Figure 13 Effect of *in vivo* cimetidine on testosterone
7α-hydroxylase activity. Microsomes were isolated
from uninduced, phenobarbital-induced and
dexamethasone-induced rats. Results are expressed
as the mean ± SEM. The sample size (N) was 8 rats
per group, except for the uninduced, cimetidine-
treated group (N = 7) and the dexamethasone-
induced, cimetidine-treated group (N = 5).
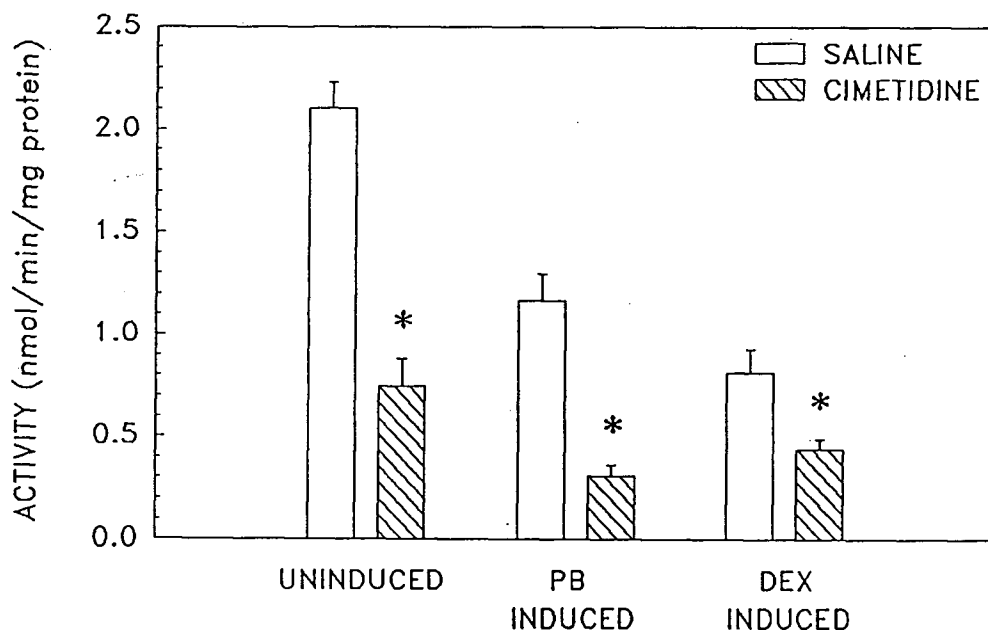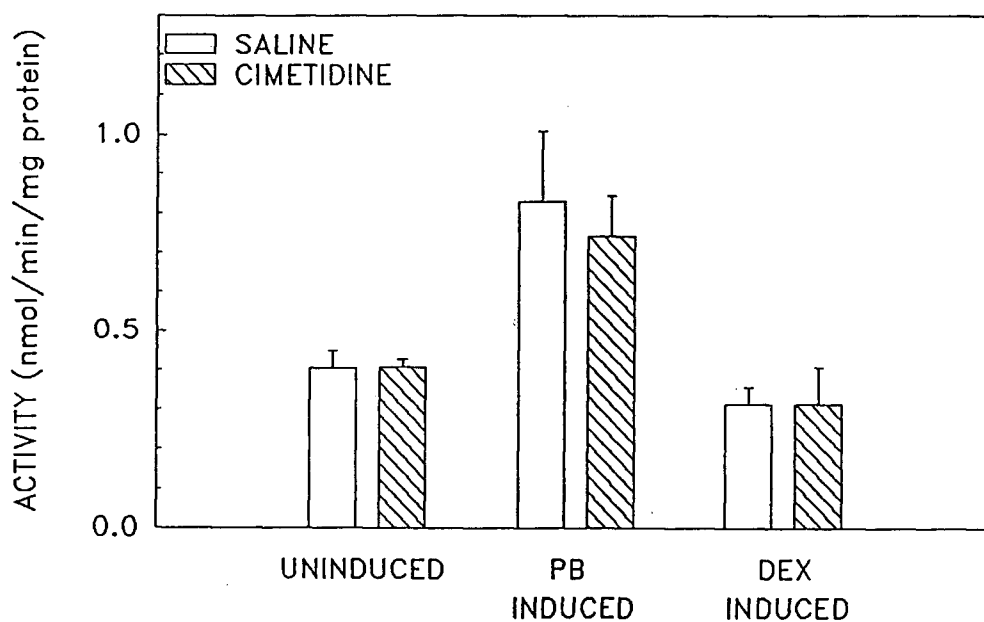
TABLE 14

EFFECT OF PHENOBARBITAL AND DEXAMETHASONE ON
TESTOSTERONE 7α-HYDROXYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	0.45 ± 0.08
Phenobarbital	8	0.83 ± 0.18
2% Tween 80	4	0.35 ± 0.08
Dexamethasone	8	0.31 ± 0.04

Results are expressed as the mean ± SEM for the number
(N) of rats indicated.

3.1.6.4   Testosterone 16α-Hydroxylase Activity

In vivo cimetidine inhibited testosterone 16α-hydroxylase activity by 60% in microsomes from uninduced rats (Figure 14). This activity was increased 2.1-fold by phenobarbital, but was decreased 40% by dexamethasone (Table 15). *In vivo* cimetidine did not affect this activity in microsomes from either phenobarbital- or dexamethasone-induced rats (Figure 14).

Cytochrome P450IIC11 accounts for more than 85% of the testosterone 16α-hydroxylase activity in hepatic microsomes from uninduced adult male rats (Waxman, 1984; Waxman *et al.*, 1987). This enzyme is suppressed in livers of rats pretreated with phenobarbital or dexamethasone. It has been shown that cytochrome P450IIC11 is not the major enzyme responsible for testosterone 16α-hydroxylase activity in hepatic microsomes from phenobarbital-induced adult male rats (Waxman, 1984; Waxman *et al.*, 1987). In this case, the major contributors are cytochromes P450IIB1/2 (Thomas *et al.*, 1981; Reik *et al.*, 1985; Waxman *et al.*, 1987). However, it has not yet been determined which cytochrome P-450 enzymes contribute to this activity in microsomes from dexamethasone-induced male rats.

Figure 14 Effect of *in vivo* cimetidine on testosterone
16α-hydroxylase activity. Microsomes were
isolated from uninduced, phenobarbital-induced and
dexamethasone-induced rats. Results are expressed
as the mean ± SEM. The sample size (N) was 8 rats
per group, except for the uninduced, cimetidine-
treated group (N = 7) and the dexamethasone-
induced, cimetidine-treated group (N = 5).
*p < 0.001, compared to the saline-treated group.

TABLE 15

EFFECT OF PHENOBARBITAL AND DEXAMETHASONE ON
TESTOSTERONE 16α-HYDROXYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	3.72 ± 0.53
Phenobarbital	8	7.78 ± 0.79[#]
2% Tween 80	4	3.04 ± 0.50
Dexamethasone	8	1.81 ± 0.21[*]

Results are expressed as the mean $\pm$ SEM for the number (N) of rats indicated. [*]$p < 0.025$, compared to the corresponding control group. [#]$p < 0.01$, compared to the corresponding control group.

3.1.6.5   Testosterone 16β-Hydroxylase Activity

Only trace levels (less than 0.1 nmol/min/mg protein) of the 16β-hydroxytestosterone metabolite were formed by microsomes from uninduced rats (Table 16) and accurate quantitation of this metabolite was not possible.   As a result, the effect of *in vivo* cimetidine on this activity in microsomes from uninduced rats was not determined.

Phenobarbital pretreatment increased testosterone 16β-hydroxylase activity by at least 48-fold (Table 16).   *In vivo* cimetidine did not inhibit this activity in microsomes from phenobarbital-induced rats (Figure 15).   It has been shown that cytochromes P450IIB1/2 account for more than 90% of the testosterone 16β-hydroxylase activity in hepatic microsomes from phenobarbital-induced rats (Reik *et al.*, 1985; Waxman *et al.*, 1987).

A possible explanation for the lack of effect of cimetidine on testosterone 16β-hydroxylase activity could be that the inhibition by cimetidine was competitive and the substrate concentration used in the assay was too high for inhibition to be detected.   Therefore, the effect of *in vivo* cimetidine on testosterone 16β-hydroxylase activity was determined at lower substrate concentrations.   However, even at 1/25th of the usual substrate concentration, *in vivo* cimetidine did not inhibit testosterone 16β-hydroxylase

TABLE 16

EFFECT OF PHENOBARBITAL AND DEXAMETHASONE ON
TESTOSTERONE 16β-HYDROXYLASE ACTIVITY

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	< 0.1
Phenobarbital	8	4.82 ± 0.45
2% Tween 80	4	< 0.1
Dexamethasone	8	0.53 ± 0.04

Results are expressed as the mean ± SEM for the number (N) of rats indicated. Statistical analysis was not performed on these data.

Figure 15 Effect of *in vivo* cimetidine on testosterone
16β-hydroxylase activity. Microsomes were
isolated from phenobarbital-induced and
dexamethasone-induced rats. Results are expressed
as the mean ± SEM. The sample size (N) was 8 rats
per group, except for the uninduced, cimetidine-
treated group (N = 7) and the dexamethasone-
induced, cimetidine-treated group (N = 5).
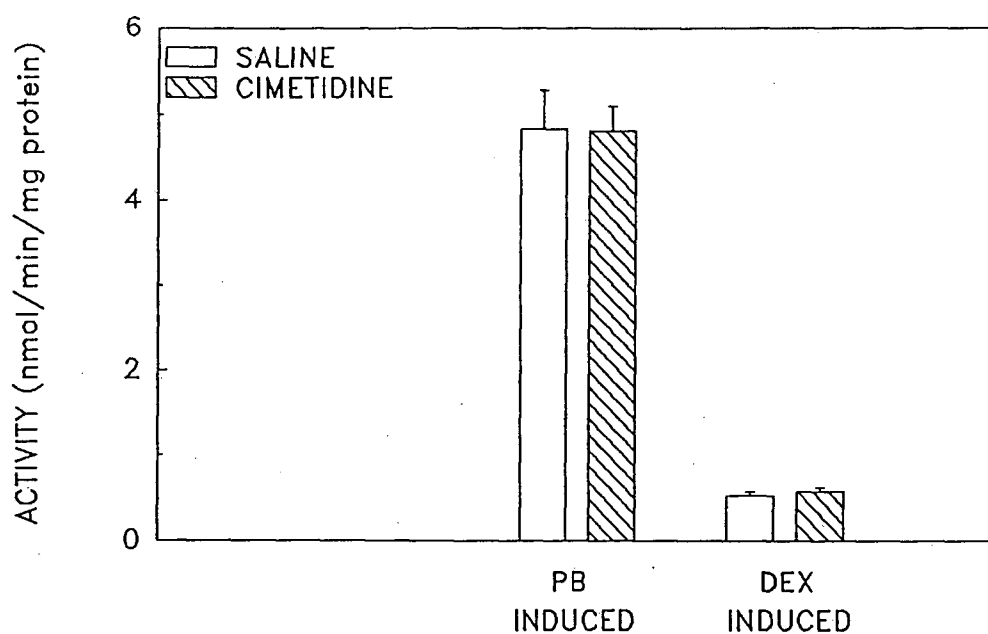
activity in microsomes from phenobarbital-induced rats (Figure 16).

Dexamethasone pretreatment increased testosterone 16β-hydroxylase activity by at least 5-fold (Table 16). *In vivo* cimetidine also did not inhibit this activity in microsomes from dexamethasone-induced rats (Figure 15). It is not known which cytochrome P-450 enzymes contribute to testosterone 16β-hydroxylase activity in microsomes from dexamethasone-induced rats.

3.1.6.6 Androstenedione Formation

In vivo cimetidine inhibited androstenedione formation by 31% in microsomes from uninduced rats (Figure 17). The activity was increased 2.8-fold by phenobarbital pretreatment, but was decreased 40% following dexamethasone pretreatment (Table 17). *In vivo* cimetidine did not affect androstenedione formation in microsomes from phenobarbital- or dexamethasone-induced rats (Figure 17).

Cytochromes P450IIB1/2 account for 60-70% of the formation of androstenedione in hepatic microsomes from phenobarbital-treated adult male rats (Reik *et al.*, 1985). It is not known which enzymes are responsible for the remainder of the activity in microsomes from this group of rats. As well, it has yet to be shown which cytochrome P-450 enzymes are involved in androstenedione formation in

Figure 16 Lineweaver-Burk plot for the effect of *in vivo* cimetidine on testosterone 16β-hydroxylase activity. Phenobarbital-induced rats were sacrificed 90 min after a single injection of cimetidine HCl (150 mg/kg) or saline (control). Microsomes were prepared from a pool of four livers in each group. The enzyme activity (V) was determined at various substrate [S] concentrations. The symbols indicate values of the transformed data and the lines were generated by linear regression analysis.
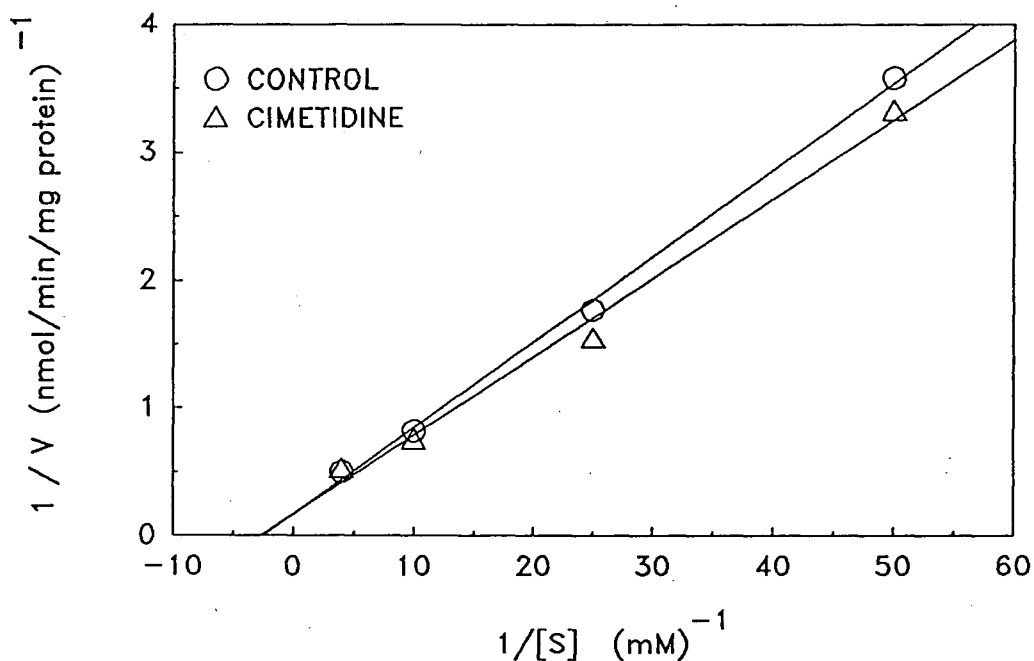
Figure 17 Effect of *in vivo* cimetidine on androstenedione formation. Microsomes were isolated from uninduced, phenobarbital-induced and dexamethasone-induced rats. Results are expressed as the mean ± SEM. The sample size (N) was 8 rats per group, except for the uninduced, cimetidine-treated group (N = 7) and the dexamethasone-induced, cimetidine-treated group (N = 5). *p < 0.001, compared to the corresponding saline-treated group.
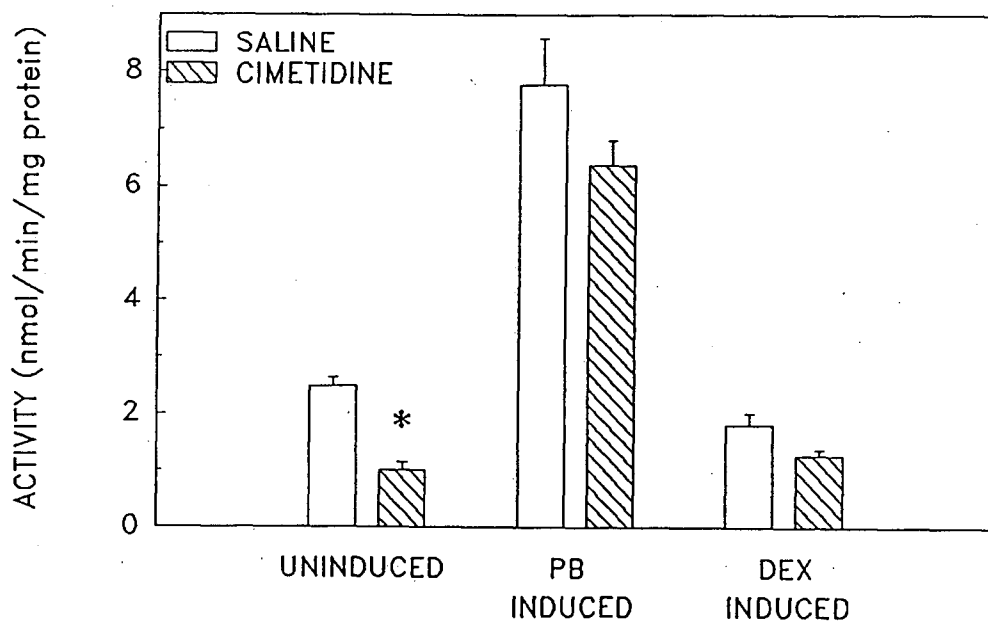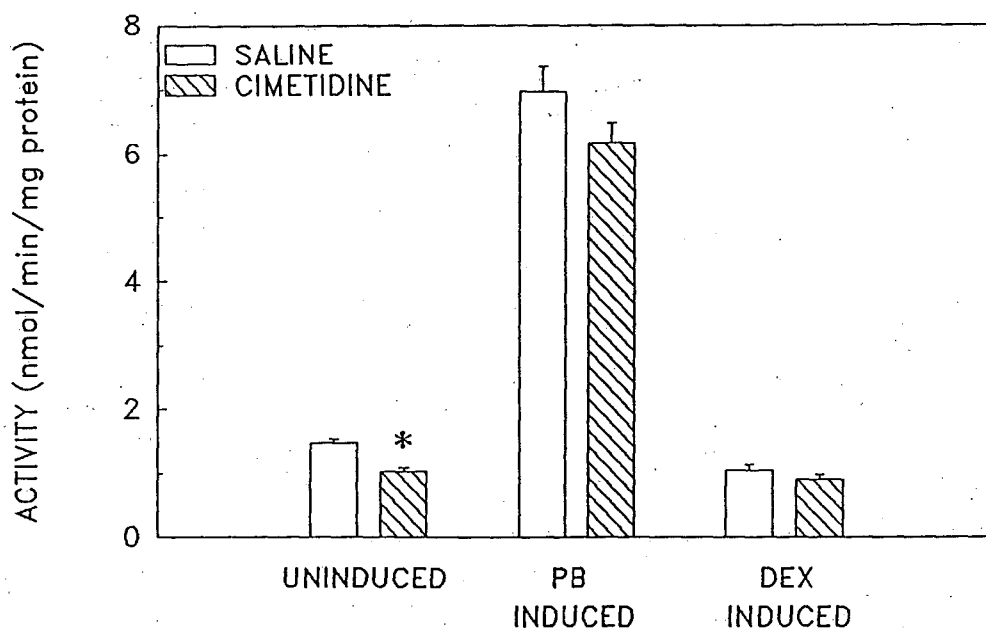
TABLE 17

EFFECT OF PHENOBARBITAL AND DEXAMETHASONE ON ANDROSTENEDIONE FORMATION

Pretreatment	N	Activity (nmol/min/mg protein)
Saline	4	2.45 ± 0.10
Phenobarbital	8	$6.97 \pm 0.39^*$
2% Tween 80	4	1.88 ± 0.29
Dexamethasone	8	$1.04 \pm 0.10^\#$

Results are expressed as the mean $\pm$ SEM for the number (N) of rats indicated. $^\#p < 0.005$, compared to the corresponding control group. $^*p < 0.001$, compared to the corresponding control group.

microsomes from uninduced or dexamethasone-induced adult male rats.

3.1.6.7   Summary

The effects of *in vivo* cimetidine on testosterone oxidation by microsomes from uninduced, phenobarbital-induced and dexamethasone-induced rats are summarized in Figure 18. Testosterone 2α- and 16α-hydroxylase activities in microsomes from the uninduced rats were inhibited by *in vivo* cimetidine. The inhibition observed in these two cases, in conjunction with the known enzyme-specificity of these two activities, indicated that *in vivo* cimetidine has an inhibitory effect on cytochrome P450IIC11. In the other cases where inhibition of testosterone oxidation by cimetidine was observed, the enzyme-specificity of the activities was unknown. Furthermore, the lack of an effect of *in vivo* cimetidine on testosterone 2β, 6β, 7α or 16β-hydroxylase activity suggests that cytochromes P450IIIA1/2, cytochrome P450IIA1 and cytochromes P450IIB1/2 are not inhibited by cimetidine.

Figure 18 Summary of the effects of *in vivo* cimetidine on testosterone oxidation. Results are based on the data from Figures 10-15 and 17. $^*p < 0.001$, compared to the saline-treated control group.
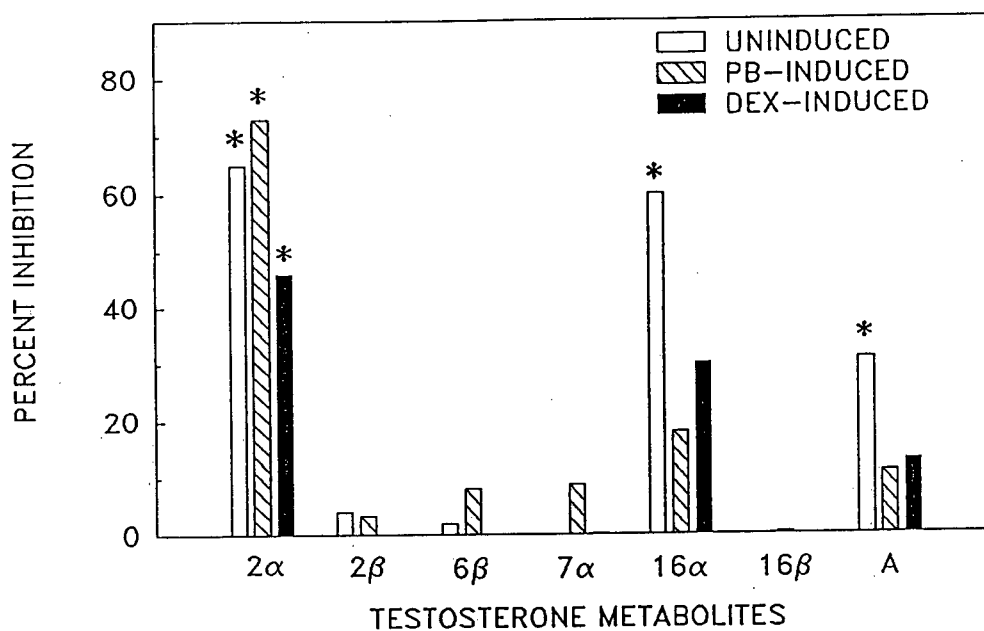
3.2 IMMUNOINHIBITION STUDIES WITH MONOSPECIFIC ANTI-CYTOCHROME P450IIC11 ANTIBODY

The major purpose of the immunoinhibition studies was to determine the role of cytochrome P450IIC11 in the microsomal enzyme activities that were inhibited by *in vivo* cimetidine; that is, aminopyrine N-demethylase, pentoxyresorufin 0-dealkylase, erythromycin N-demethylase, testosterone 2α-hydroxylase, testosterone 16α-hydroxylase and androstenedione formation. Testosterone 2β-, 6β-, 7α- and 16β-hydroxylase activities were also determined in the presence or absence of the antibody.

3.2.1 Aminopyrine N-Demethylase Activity

At saturating concentrations, the antibody inhibited aminopyrine N-demethylase activity by approximately 35% in microsomes from uninduced adult male rats (Figure 19). In contrast, there was little or no inhibitory effect of the antibody on this activity in microsomes from phenobarbital-induced rats.

3.2.2 Pentoxyresorufin 0-Dealkylase Activity

At the highest concentration used, the antibody inhibited pentoxyresorufin 0-dealkylase activity by more than 90% in microsomes from uninduced rats (Figure 20). In contrast, there was little or no inhibitory effect of the

Figure 19 Effect of monospecific anti-cytochrome P450IIC11
antibody on aminopyrine N-demethylase activity.
Microsomes were isolated from a pool of livers
from either four uninduced or phenobarbital-
induced rats. Microsomes (0.3 nmol of total
cytochrome P-450) were preincubated for 10 min at
room temperature with 0.25, 0.5, 1, 2.5, 5 or 10
mg of anti-cytochrome P450IIC11 antibody or
control IgG per nmol of total cytochrome P-450.
Enzyme activity was determined as described under
"Materials and Methods". Results are expressed as
a percent of control activity.
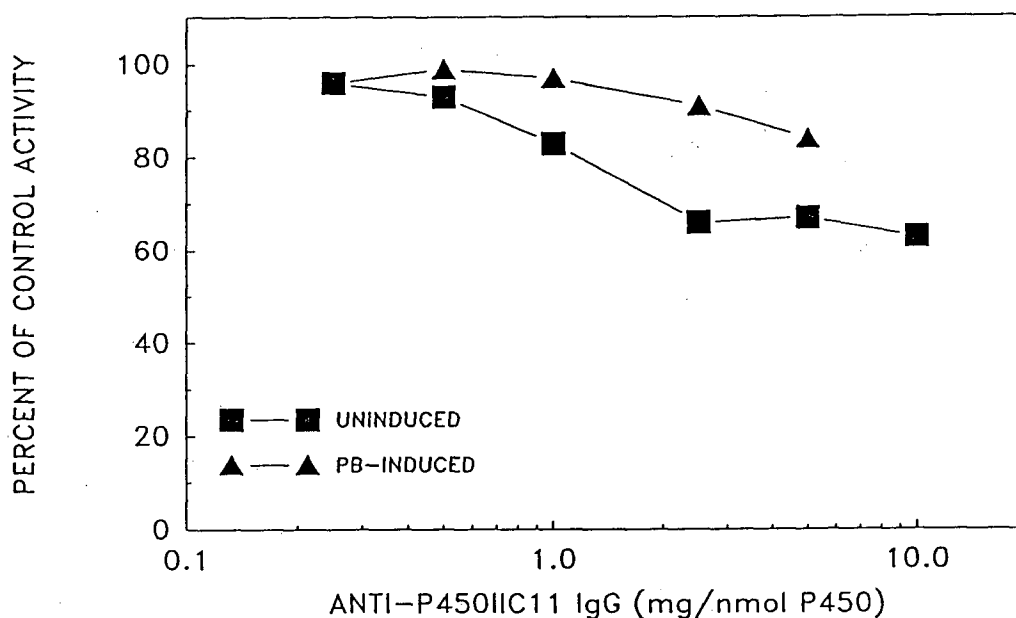
Figure 20 Effect of monospecific anti-cytochrome P450IIC11
antibody on pentoxyresorufin O-dealkylase
activity. Microsomes were isolated from a pool of
livers from either four uninduced or
phenobarbital-induced rats. Microsomes (0.3 nmol
of total cytochrome P-450) were preincubated for
10 min at room temperature with 0.25, 0.5, 1, 2.5,
5 or 10 mg of anti-cytochrome P450IIC11 antibody
or control IgG per nmol of total cytochrome P-450.
Enzyme activity was determined as described under
"Materials and Methods". Results are expressed as
a percent of control activity.

antibody on this activity in microsomes from phenobarbital-induced rats.

3.2.3 Erythromycin N-Demethylase Activity

At the concentrations used, the antibody had little or no inhibitory effect on erythromycin N-demethylase activity in microsomes from uninduced rats (Figure 21).

3.2.4 Testosterone 2α-Hydroxylase Activity

At saturating concentrations, the antibody completely inhibited testosterone 2α-hydroxylase activity in microsomes from uninduced adult male rats (Figure 22). This observation is consistent with published data (Waxman, 1984; Waxman et al., 1987) and confirms that cytochrome P450IIC11 is the enzyme responsible for testosterone 2α-hydroxylase activity in microsomes from uninduced adult male rats.

At saturating concentrations, the antibody also completely inhibited this activity in microsomes from phenobarbital-induced and dexamethasone-induced adult male rats (Figure 22). These results indicate that cytochrome P450IIC11 remains the enzyme responsible for microsomal testosterone 2α-hydroxylase activity in these groups of rats.

Figure 21 Effect of monospecific anti-cytochrome P450IIC11 antibody on erythromycin N-demethylase activity. Microsomes were isolated from a pool of livers from four uninduced rats. Microsomes (0.3 nmol of total cytochrome P-450) were preincubated for 10 min at room temperature with 0.25, 0.5, 1, 2.5, 5 or 10 mg of anti-cytochrome P450IIC11 antibody or control IgG per nmol of total cytochrome P-450. Enzyme activity was determined as described under "Materials and Methods". Results are expressed as a percent of control activity.

Figure 22 Effect of monospecific anti-cytochrome P450IIC11
antibody on testosterone 2α-hydroxylase activity.
Microsomes were isolated from a pool of livers
from four uninduced, phenobarbital-induced or
dexamethasone-induced rats. Microsomes (0.3 nmol
of total cytochrome P-450) were preincubated for
10 min at room temperature with 0.25, 0.5, 1, 2.5,
5 or 10 mg of anti-cytochrome P450IIC11 antibody
or control IgG per nmol of total cytochrome P-450.
Enzyme activity was determined as described under
"Materials and Methods". Results are expressed as
a percent of control activity.
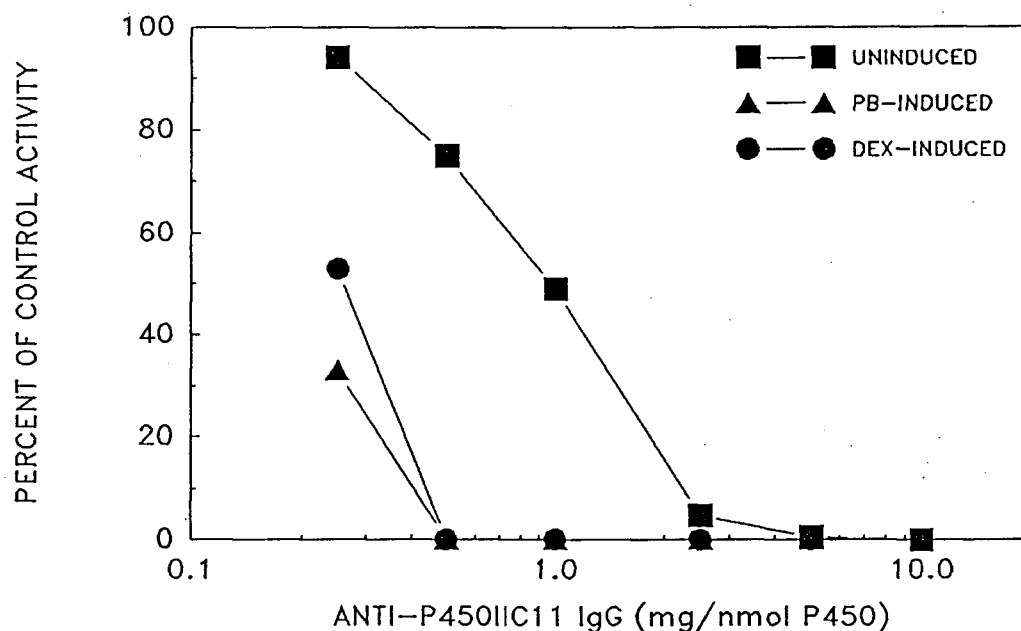
3.2.5 Testosterone 16α-Hydroxylase Activity

At saturating concentrations, the antibody inhibited testosterone 16α-hydroxylase activity by approximately 95% in microsomes from uninduced adult male rats (Figure 23). This is consistent with published data (Waxman, 1984; Waxman et al., 1987) and confirms that cytochrome P450IIC11 is the enzyme responsible for microsomal testosterone 16α-hydroxylase activity in this group of rats.

As shown in Figure 23, at saturating concentrations, the antibody had little or no inhibitory effect on this activity in microsomes from phenobarbital-induced adult male rats. It has been shown that cytochrome P450IIC11 is not the major contributor to testosterone 16α-hydroxylase activity in microsomes from phenobarbital-induced adult male rats (Waxman, 1984; Waxman et al., 1987).

At saturating concentrations, the antibody inhibited testosterone 16α-hydroxylase activity by approximately 65% in microsomes from dexamethasone-treated rats (Figure 23), indicating that cytochrome P450IIC11 partially contributes to microsomal testosterone 16α-hydroxylase activity in this group of rats.

3.2.6 Androstenedione Formation

At saturating concentrations, the antibody inhibited androstenedione formation by approximately 60% in microsomes from uninduced adult male rats (Figure 24). In contrast, it

Figure 23 Effect of monospecific anti-cytochrome P450IIC11
antibody on testosterone 16α-hydroxylase activity.
Microsomes were isolated from a pool of livers
from four uninduced, phenobarbital-induced or
dexamethasone-induced rats. Microsomes (0.3 nmol
of total cytochrome P-450) were preincubated for
10 min at room temperature with 0.25, 0.5, 1, 2.5,
5 or 10 mg of anti-cytochrome P450IIC11 antibody
or control IgG per nmol of total cytochrome P-450.
Enzyme activity was determined as described under
"Materials and Methods". Results are expressed as
a percent of control activity.
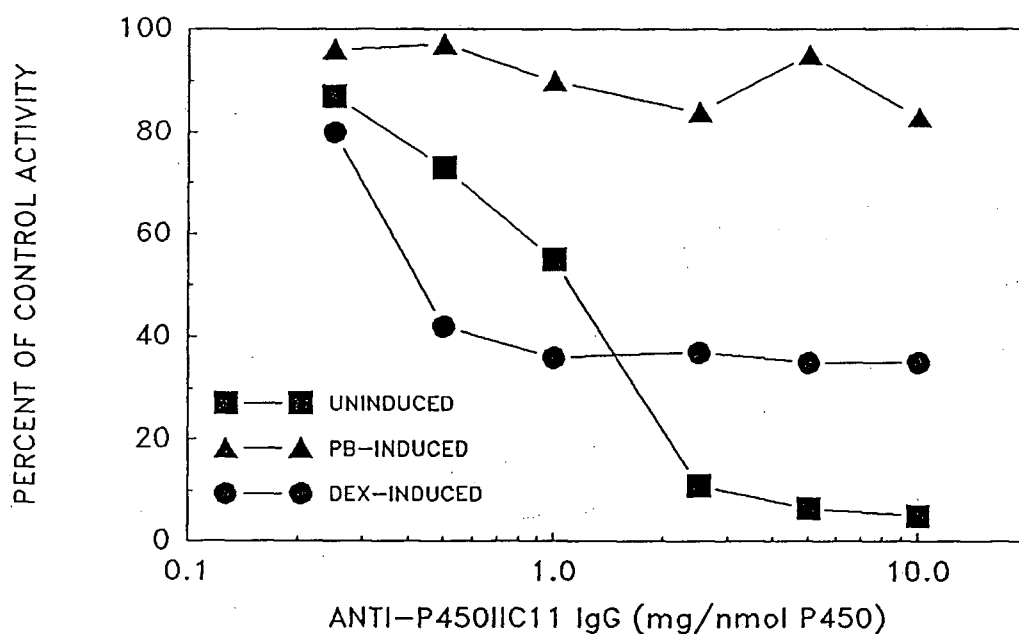
Figure 24 Effect of monospecific anti-cytochrome P450IIC11 antibody on androstenedione formation. Microsomes were isolated from a pool of livers from four uninduced, phenobarbital-induced or dexamethasone-induced rats. Microsomes (0.3 nmol of total cytochrome P-450) were preincubated for 10 min at room temperature with 0.25, 0.5, 1, 2.5, 5 or 10 mg of anti-cytochrome P450IIC11 antibody or control IgG per nmol of total cytochrome P-450. Enzyme activity was determined as described under "Materials and Methods". Results are expressed as a percent of control activity.
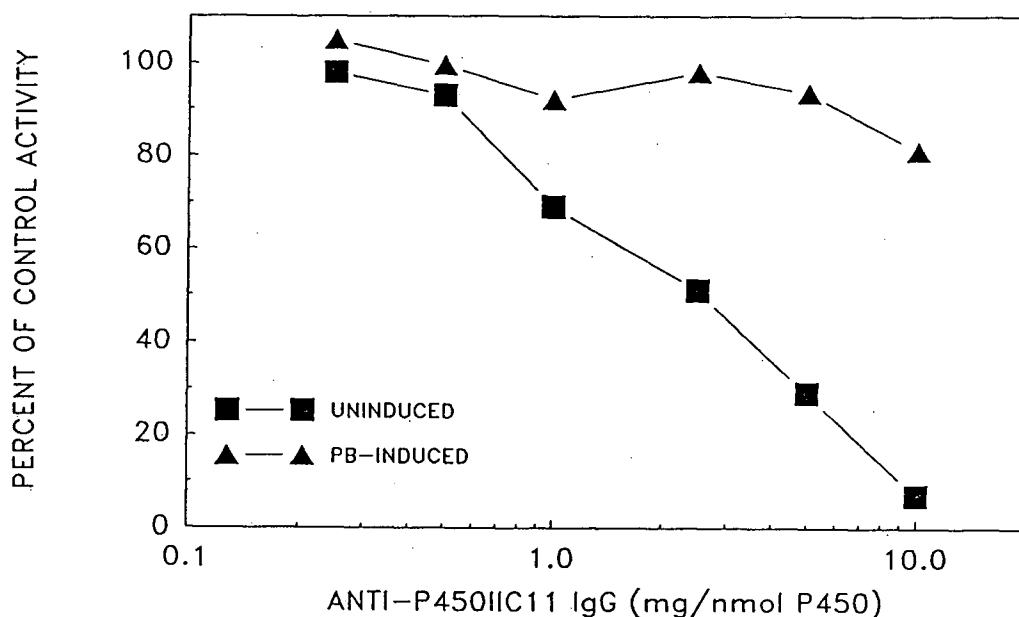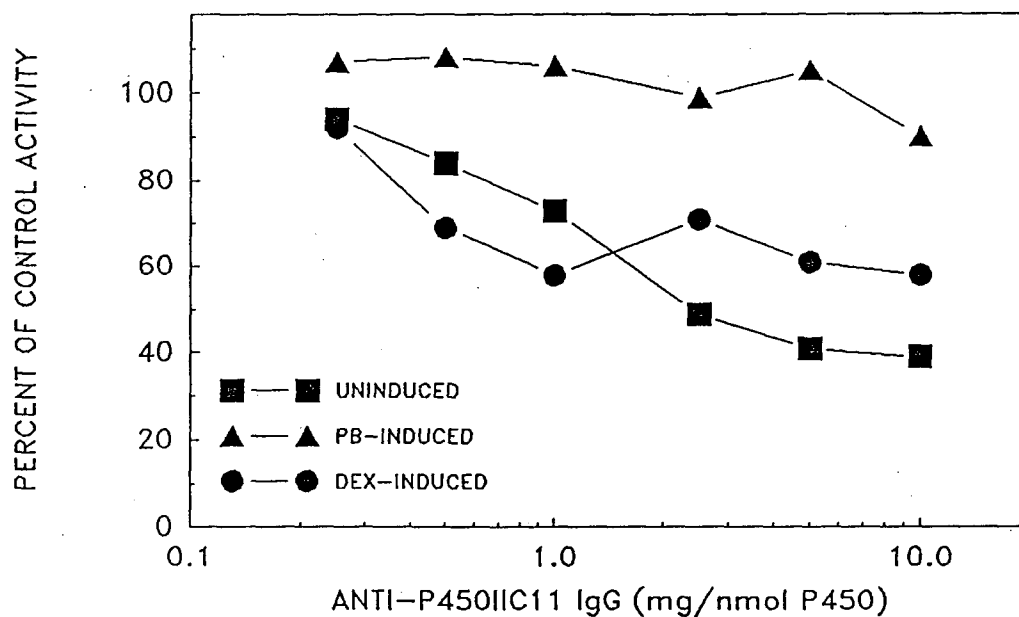
had little or no inhibitory effect on this reaction in microsomes from phenobarbital-induced adult male rats. In microsomes from dexamethasone-induced adult male rats, the antibody inhibited this reaction by approximately 40%.

3.2.7 Testosterone 2β-, 6β-, 7α- and 16β-Hydroxylase Activities

The antibody did not inhibit the cytochrome P450IIIA1/2-mediated testosterone 2β- and 6β-hydroxylase activities or the cytochrome P450IIA1-mediated testosterone 7α-hydroxylase activity in microsomes from uninduced, phenobarbital- or dexamethasone-induced rats (Figures 25-27). It also did not inhibit the cytochrome P450IIB1/2-mediated testosterone 16β-hydroxylase activity in microsomes from phenobarbital-induced rats (Figure 28).

Figure 25 Effect of monospecific anti-cytochrome P450IIC11 antibody on testosterone 2β-hydroxylase activity. Microsomes were isolated from a pool of livers from four uninduced, phenobarbital-induced or dexamethasone-induced rats. Microsomes (0.3 nmol of total cytochrome P-450) were preincubated for 10 min at room temperature with 0.25, 0.5, 1, 2.5, 5 or 10 mg of anti-cytochrome P450IIC11 antibody or control IgG per nmol of total cytochrome P-450. Enzyme activity was determined as described under "Materials and Methods". Results are expressed as a percent of control activity.
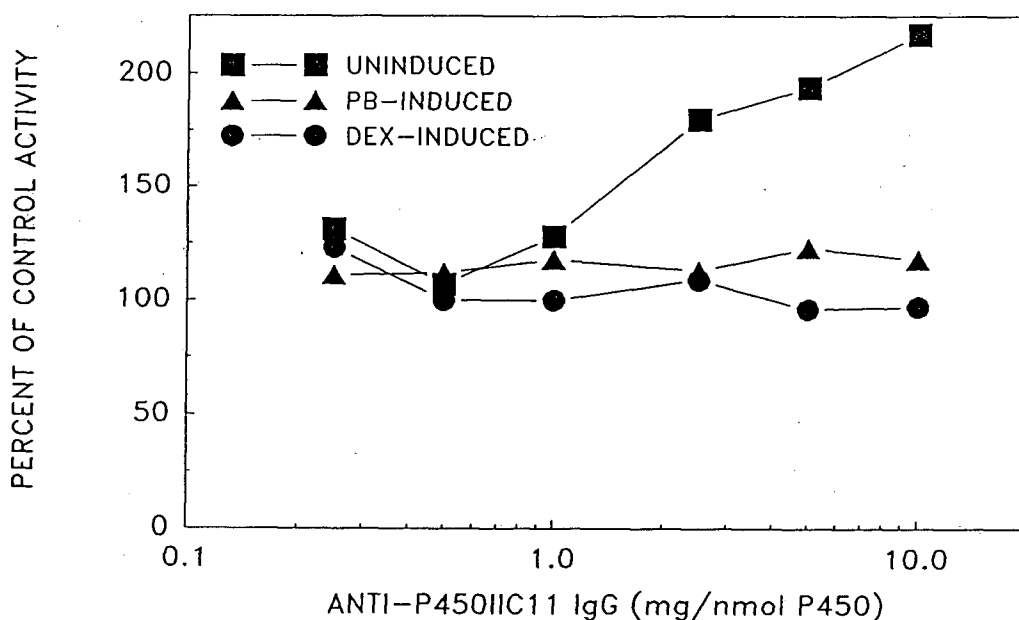
Figure 26 Effect of monospecific anti-cytochrome P450IIC11
antibody on testosterone 6β-hydroxylase activity.
Microsomes were isolated from a pool of livers
from four uninduced, phenobarbital-induced or
dexamethasone-induced rats. Microsomes (0.3 nmol
of total cytochrome P-450) were preincubated for
10 min at room temperature with 0.25, 0.5, 1, 2.5,
5 or 10 mg of anti-cytochrome P450IIC11 antibody
or control IgG per nmol of total cytochrome P-450.
Enzyme activity was determined as described under
"Materials and Methods". Results are expressed as
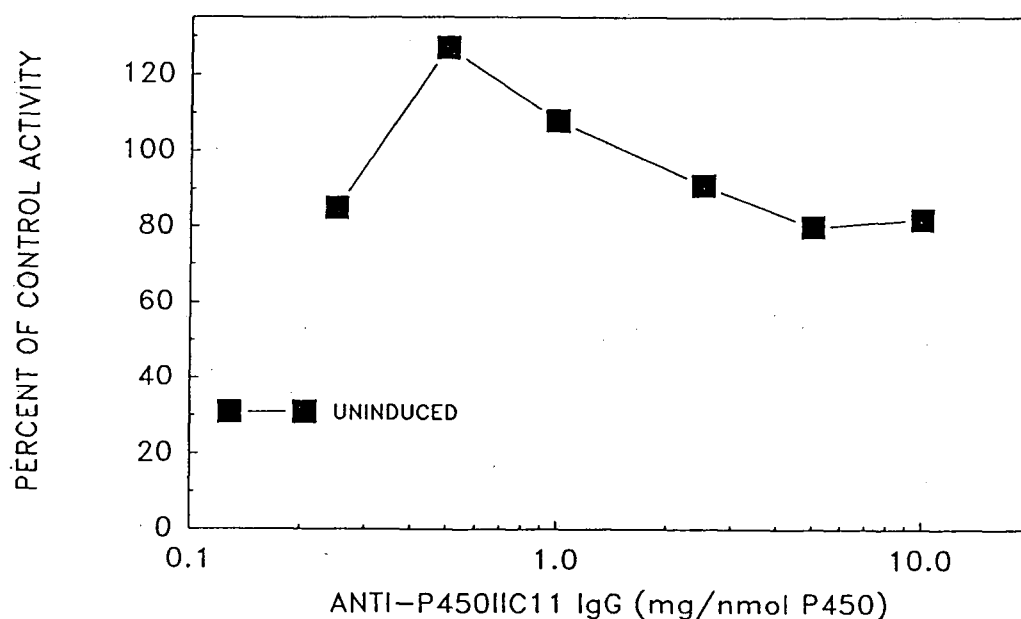a percent of control activity.
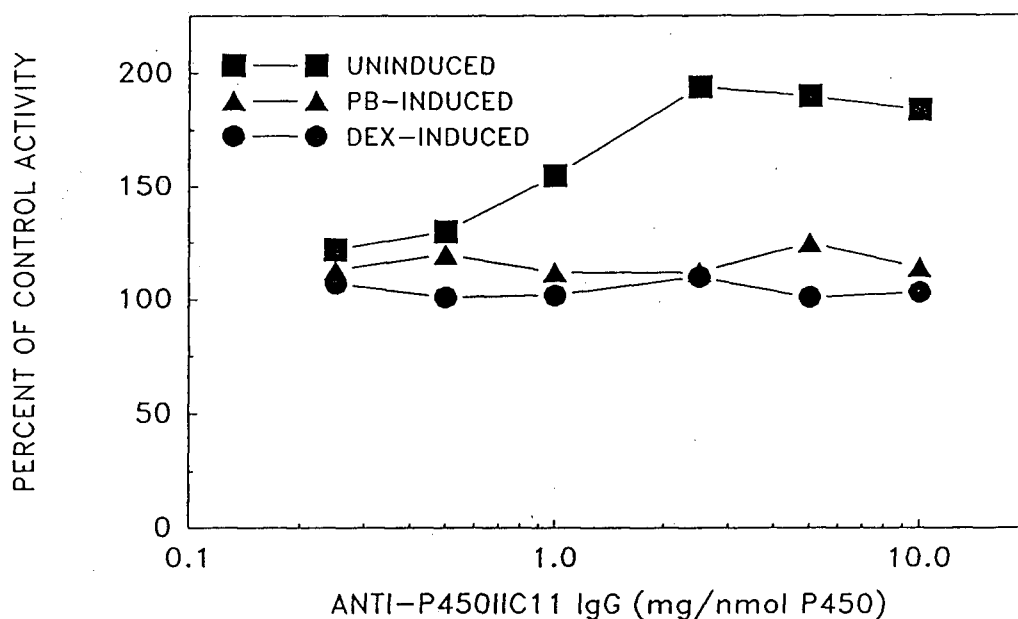
Figure 27 Effect of monospecific anti-cytochrome P450IIC11
antibody on testosterone 7α-hydroxylase activity.
Microsomes were isolated from a pool of livers
from four uninduced, phenobarbital-induced or
dexamethasone-induced rats. Microsomes (0.3 nmol
of total cytochrome P-450) were preincubated for
10 min at room temperature with 0.25, 0.5, 1, 2.5,
5 or 10 mg of anti-cytochrome P450IIC11 antibody
or control IgG per nmol of total cytochrome P-450.
Enzyme activity was determined as described under
"Materials and Methods". Results are expressed as
a percent of control activity.
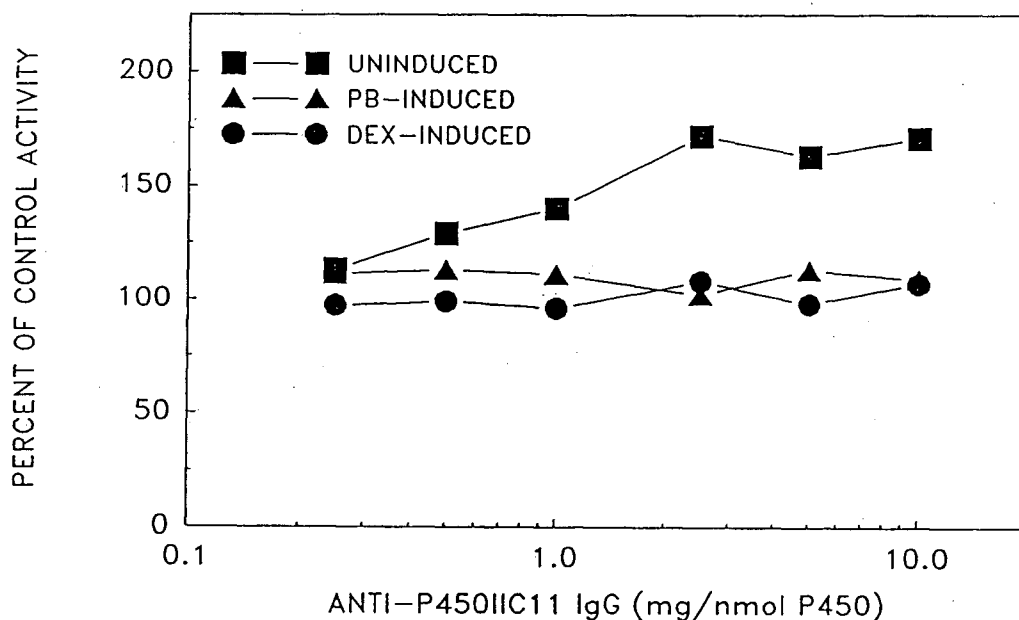
Figure 28 Effect of monospecific anti-cytochrome P450IIC11
antibody on testosterone 16β–hydroxylase activity.
Microsomes were isolated from a pool of livers
from four phenobarbital-induced rats. Microsomes
(0.3 nmol of total cytochrome P-450) were
preincubated for 10 min at room temperature with
0.25, 0.5, 1, 2.5, 5 or 10 mg of anti-cytochrome
P450IIC11 antibody or control IgG per nmol of
total cytochrome P-450.   Enzyme activity was
determined as described under "Materials and
Methods".   Results are expressed as a percent of
control activity.
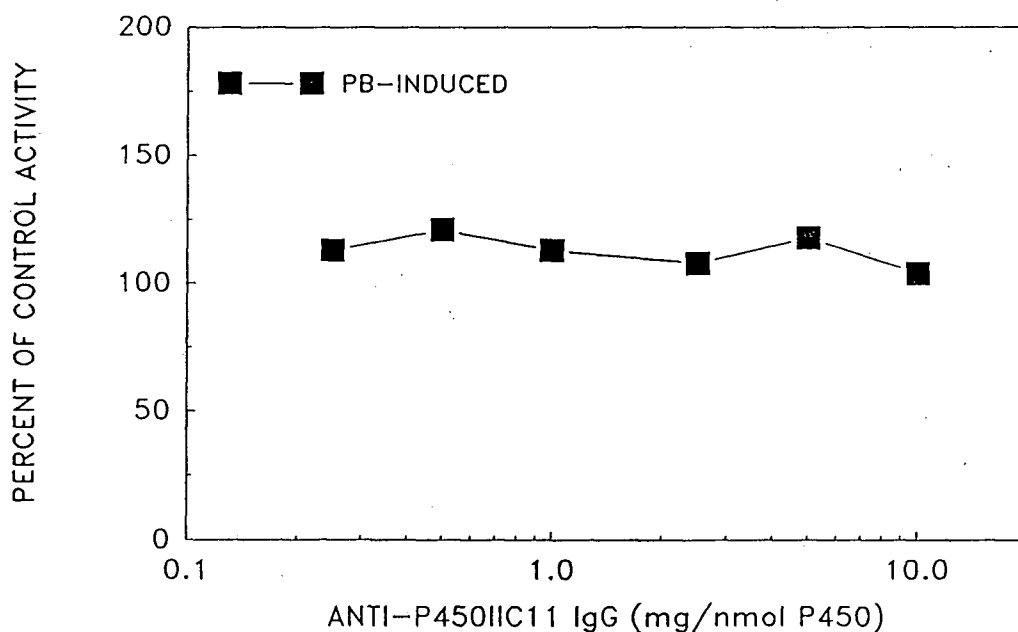
3.3   STUDIES WITH *IN VITRO* CIMETIDINE

The results presented in the foregoing sections indicated that *in vivo* cimetidine inhibits hepatic cytochrome P450IIC11, but apparently not cytochrome P450IIA1, cytochromes P450IIB1/2 or cytochromes P450IIIA1/2 in the adult male rat.   Experiments were performed to determine whether the same results would be obtained with *in vitro* cimetidine, using the same enzyme activities as in the studies with *in vivo* cimetidine.

3.3.1   Pentoxyresorufin O-Dealkylase Activity

In vitro cimetidine inhibited pentoxyresorufin O-dealkylase activity in microsomes from phenobarbital-induced rats (Figure 29).   The IC_{50} value was 1.0 mM (Table 18).   This indicates that *in vitro* cimetidine inhibits cytochromes P450IIB1/2 and is in contrast to the apparent lack of inhibition of pentoxyresorufin O-dealkylase activity in microsomes from phenobarbital-induced rats by *in vivo* cimetidine (Section 3.1.3).

3.3.2   Erythromycin N-Demethylase Activity

In vitro cimetidine inhibited erythromycin N-demethylase activity in microsomes from dexamethasone-induced rats (Figure 30).   The IC_{50} value was 2.8 mM (Table 18).   This indicates that *in vitro* cimetidine

Figure 29 Effect of *in vitro* cimetidine on pentoxyresorufin
0-dealkylase activity. Livers from four
phenobarbital-induced rats were pooled and
microsomes were prepared. Cimetidine
hydrochloride (0.15 - 10 mM) or distilled water
(control) was added *in vitro*. Results are
expressed as a percent of control activity. The
activity of the control sample was 5.03
nmol/min/mg protein.
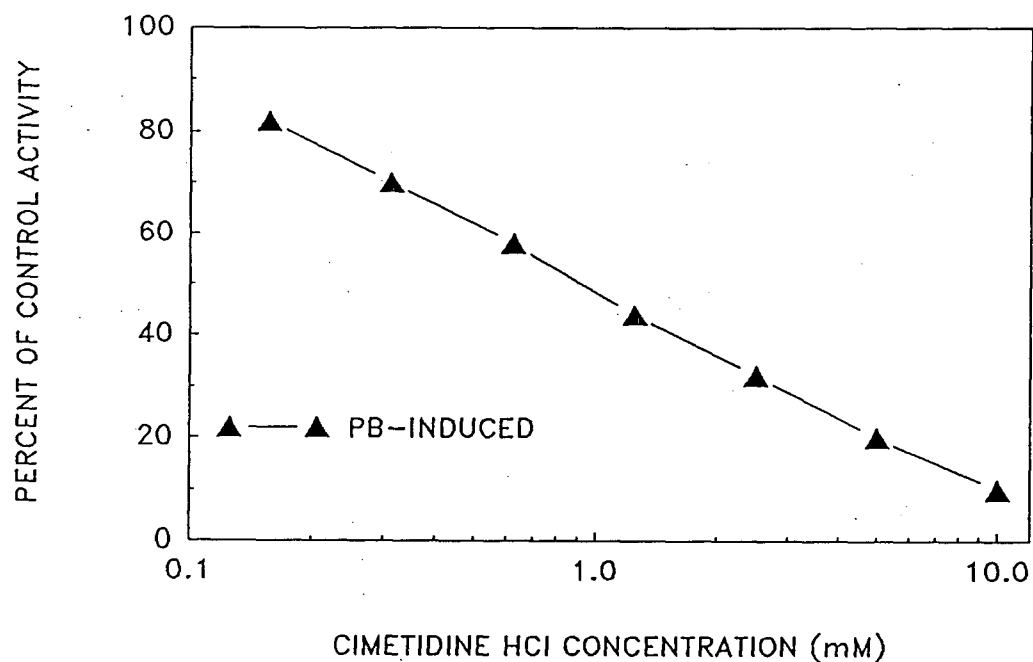
TABLE 18

IC$_{50}$ VALUES FOR THE INHIBITION OF
PENTOXYRESORUFIN O-DEALKYLASE, ERYTHROMYCIN N-DEMETHYLASE
AND ETHOXYRESORUFIN O-DEETHYLASE ACTIVITIES
BY CIMETIDINE *IN VITRO*

Enzyme Activity	Inducer	Substrate Concentration (mM)	IC$_{50}$ (mM)
Pentoxyresorufin O-Dealkylase	Phenobarbital	0.005	1.0
Erythromycin N-Demethylase	Dexamethasone	0.4	2.8
Ethoxyresorufin O-Deethylase	None	0.005	0.3
Ethoxyresorufin O-Deethylase	β-Naphtho-flavone	0.005	7.1

IC$_{50}$ values were determined graphically based on the data from Figures 29-31.

Figure 30 Effect of *in vitro* cimetidine on erythromycin
N-demethylase activity. Livers from four
dexamethasone-induced rats were pooled and
microsomes were prepared. Cimetidine
hydrochloride (0.625 - 10 mM) or distilled water
(control) was added *in vitro*. Results are
expressed as a percent of control activity. The
activity of the control sample was 10.41
nmol/min/mg protein.
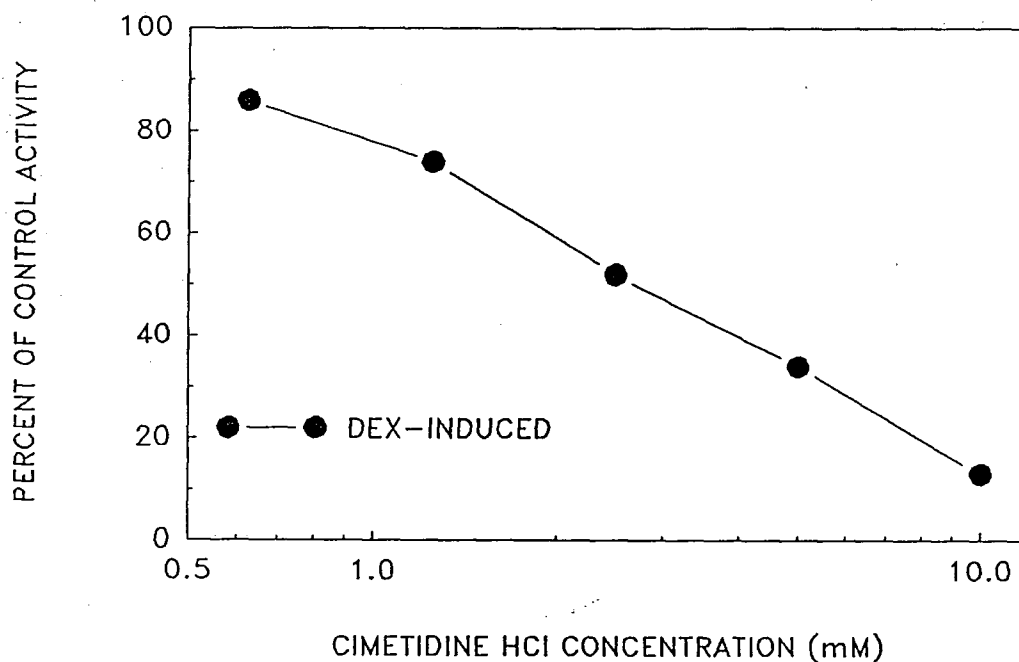
inhibits cytochromes P450IIIA1/2 and is in contrast to the apparent lack of inhibition of erythromycin N-demethylase activity in microsomes from dexamethasone-induced rats by *in vivo* cimetidine (Section 3.1.4).

3.3.3 Ethoxyresorufin O-Deethylase Activity

In vitro cimetidine inhibited ethoxyresorufin O-deethylase activity in microsomes from uninduced rats (Figure 31). The IC_{50} value was 0.3 mM (Table 18). This indicates that *in vitro* cimetidine inhibits cytochrome P450IA2 and is consistent with the observed inhibition of ethoxyresorufin O-deethylase activity in microsomes from uninduced rats by *in vivo* cimetidine (Section 3.1.5).

In vitro cimetidine also inhibited ethoxyresorufin O-deethylase activity in microsomes from β-naphthoflavone-induced rats (Figure 31). The IC_{50} value was 7.1 mM (Table 18). This is in contrast to the apparent lack of inhibition of ethoxyresorufin O-deethylase activity in microsomes from β-naphthoflavone-induced rats by *in vivo* cimetidine (Section 3.1.5).

3.3.4 Testosterone 2α-Hydroxylase Activity

In vitro cimetidine inhibited testosterone 2α-hydroxylase activity in microsomes from uninduced rats (Figure 32). The IC_{50} value was 7.4 mM (Table 19). *In vitro* cimetidine also inhibited this activity in microsomes

Figure 31 Effect of *in vitro* cimetidine on ethoxyresorufin
0-deethylase activity. Microsomes were isolated
from a pool of livers from either four uninduced
or β-naphthoflavone-induced rats. Cimetidine
hydrochloride (0.02 - 10 mM) or distilled water
(control) was added *in vitro*. Results are
expressed as a percent of control activity.
Control activity in nmol/min/mg protein:
uninduced, 0.42; β-naphthoflavone-induced, 11.71.

Figure 32 Effect of *in vitro* cimetidine on testosterone 2α-hydroxylase activity. Microsomes were isolated from a pool of livers from either four uninduced or phenobarbital-induced rats. Cimetidine hydrochloride (0.02 - 10 mM) or distilled water (control) was added *in vitro*. Results are expressed as a percent of control activity. Control activity in nmol/min/mg protein: uninduced, 1.55; phenobarbital-induced, 0.92.
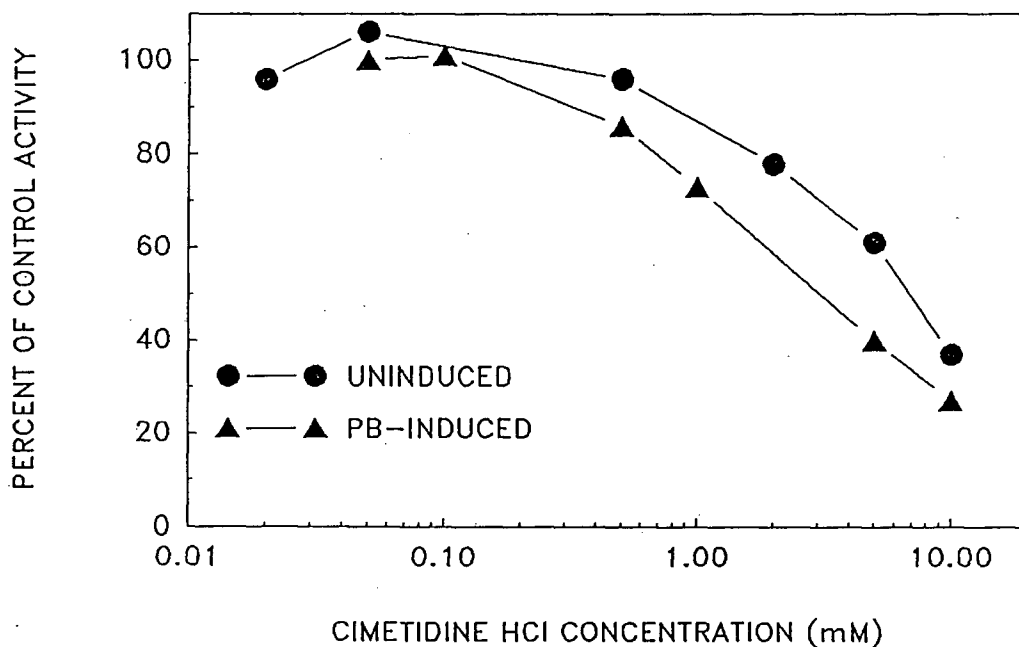
TABLE 19

IC$_{50}$ VALUES FOR THE INHIBITION OF
TESTOSTERONE OXIDATION BY CIMETIDINE *IN VITRO*

Testosterone Metabolite	Uninduced	Phenobarbital- induced
2α	7.4	3.7
2β	1.7	1.6
6β	1.7	2.2
7α	N.I.	N.I.
16α	6.4	4.2
16β	N.D.	3.3
A	> 5	5.5

The substrate concentration was 0.25 mM. IC$_{50}$ values (mM) were determined graphically based on the data from Figures 32-38. Abbreviations: N.I., no inhibition with cimetidine concentrations of up to 10 mM; N.D., not determined; A, androstenedione.

from phenobarbital-induced rats (Figure 32) and the IC_{50} value in that case was 3.7 mM (Table 19). These results indicate that *in vitro* cimetidine inhibits cytochrome P450IIC11 and are consistent with the observed inhibition of testosterone 2α-hydroxylase activity in microsomes from uninduced and phenobarbital-induced rats by *in vivo* cimetidine (Section 3.1.6.1).

3.3.5 Testosterone 2β- and 6β-Hydroxylase Activities

In vitro cimetidine inhibited testosterone 2β- and 6β-hydroxylase activities in microsomes from uninduced rats (Figures 33 and 34). The IC_{50} value was 1.7 mM in both cases (Table 19). *In vitro* cimetidine also inhibited these two activities in microsomes from phenobarbital-induced rats (Figures 33 and 34). The IC_{50} value was 1.6 mM for the inhibition of testosterone 2β-hydroxylase activity and 2.2 mM for the inhibition of testosterone 6β-hydroxylase activity (Table 19). These results indicate that *in vitro* cimetidine inhibits cytochromes P450IIIA1/2 and are in contrast to the apparent lack of inhibition of testosterone 2β- and 6β-hydroxylase activities in microsomes from uninduced and phenobarbital-induced rats by *in vivo* cimetidine (Section 3.1.6.2).

Figure 33 Effect of *in vitro* cimetidine on testosterone
2β-hydroxylase activity. Microsomes were isolated
from a pool of livers from either four uninduced
or phenobarbital-induced rats. Cimetidine
hydrochloride (0.02 - 10 mM) or distilled water
(control) was added *in vitro*. Results are
expressed as a percent of control activity.
Control activity in nmol/min/mg protein:
uninduced, 0.04; phenobarbital-induced, 0.29.
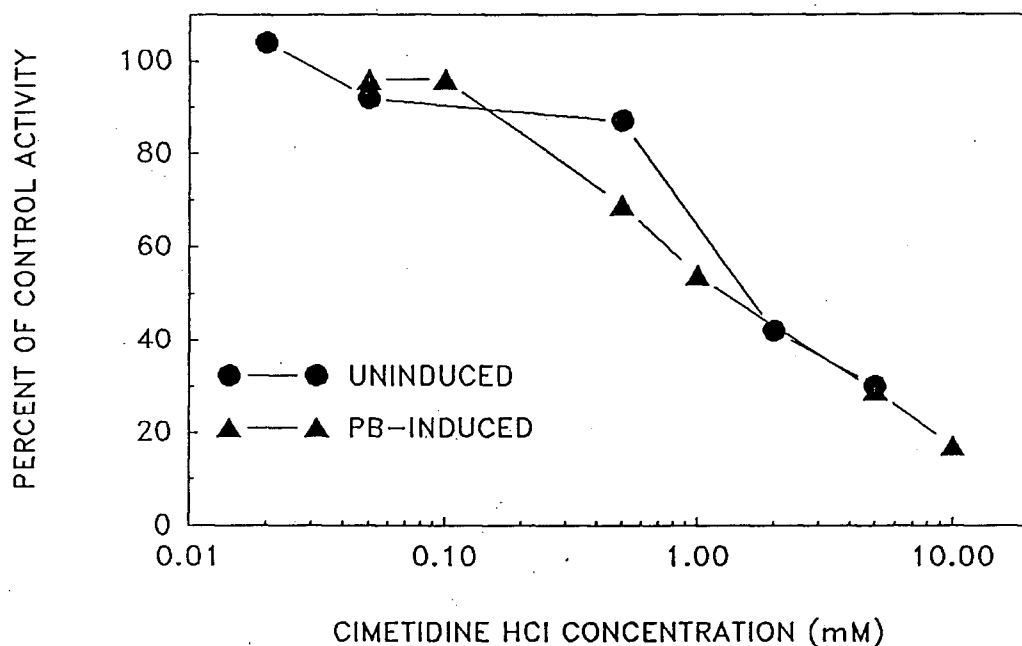
Figure 34 Effect of *in vitro* cimetidine on testosterone 6β-hydroxylase activity. Microsomes were isolated from a pool of livers from either four uninduced or phenobarbital-induced rats. Cimetidine hydrochloride (0.02 - 10 mM) or distilled water (control) was added *in vitro*. Results are expressed as a percent of control activity. Control activity in nmol/min/mg protein: uninduced, 1.55; phenobarbital-induced, 7.16.
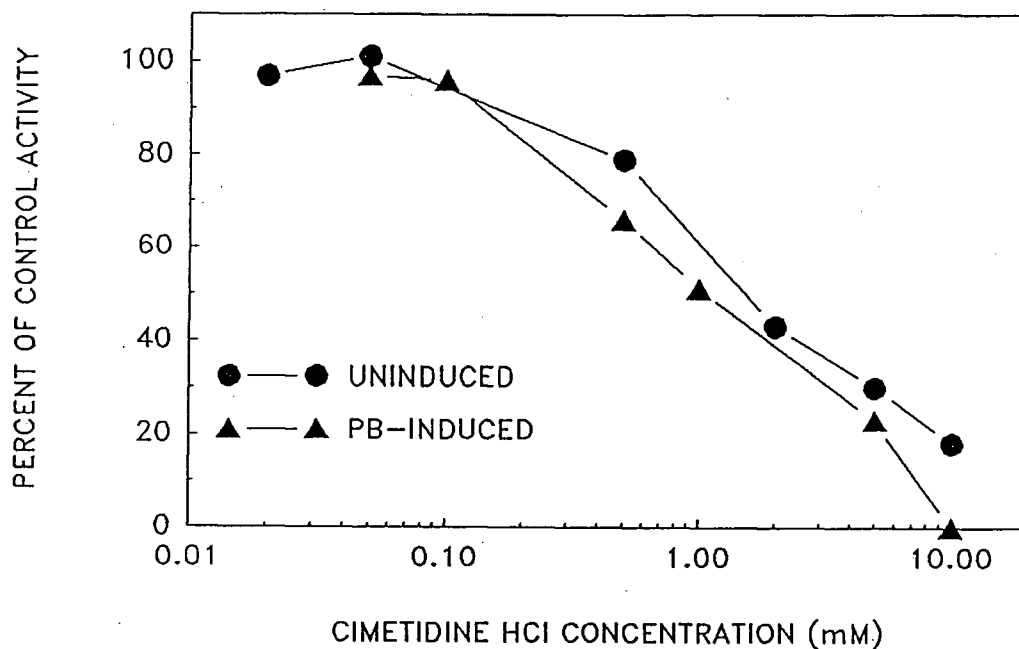
3.3.6 Testosterone 7α-Hydroxylase Activity

In vitro cimetidine, at concentrations of up to 10 mM, did not inhibit testosterone 7α-hydroxylase activity in microsomes from uninduced or phenobarbital-induced rats (Figure 35). These results suggest that *in vitro* cimetidine does not inhibit cytochrome P450IIA1 and are consistent with the apparent lack of inhibition of testosterone 7α-hydroxylase activity in microsomes from uninduced and phenobarbital-induced rats by *in vivo* cimetidine (Section 3.1.6.3).

3.3.7 Testosterone 16α-Hydroxylase Activity

In vitro cimetidine inhibited testosterone 16α-hydroxylase activity in microsomes from uninduced rats (Figure 36). The IC_{50} value was 6.4 mM (Table 19). This indicates that *in vitro* cimetidine inhibits cytochrome P450IIC11 and is consistent with the inhibition of testosterone 16α-hydroxylase activity in microsomes from uninduced rats by *in vivo* cimetidine (Section 3.1.6.4).

In vitro cimetidine also inhibited testosterone 16α-hydroxylase activity in microsomes from phenobarbital-induced rats (Figure 36). The IC_{50} value was 4.2 mM (Table 19). This is in contrast to the apparent lack of inhibition of testosterone 16α-hydroxylase activity in microsomes from phenobarbital-induced rats by *in vivo* cimetidine (Section

Figure 35 Effect of *in vitro* cimetidine on testosterone 7α-hydroxylase activity. Microsomes were isolated from a pool of livers from either four uninduced or phenobarbital-induced rats. Cimetidine hydrochloride (0.02 - 10 mM) or distilled water (control) was added *in vitro*. Results are expressed as a percent of control activity. Control activity in nmol/min/mg protein: uninduced, 0.42; phenobarbital-induced, 0.84.
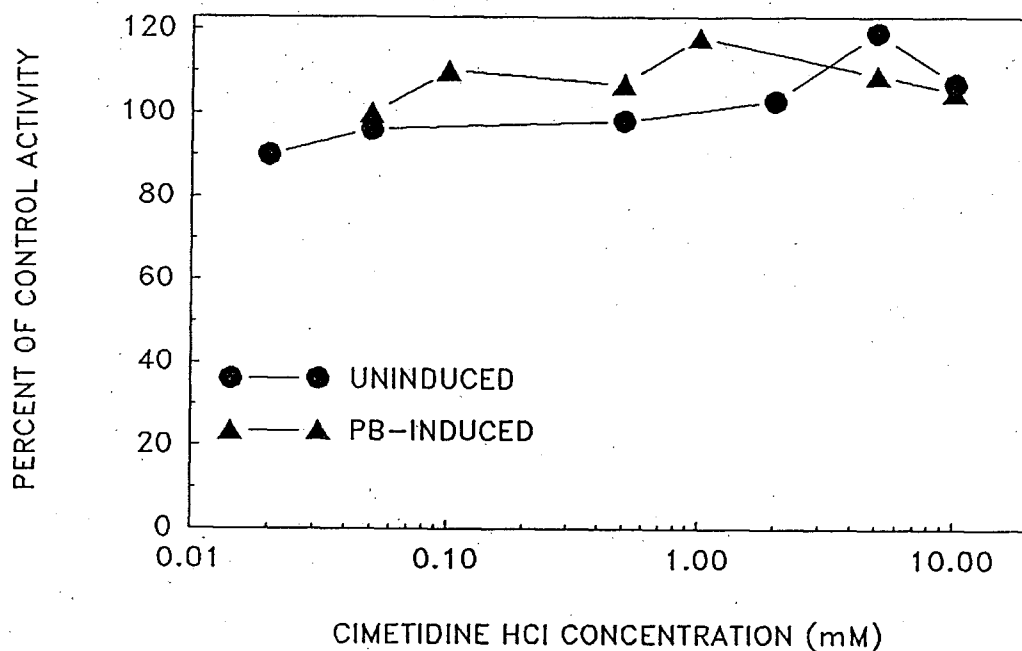
Figure 36 Effect of *in vitro* cimetidine on testosterone 16α-
hydroxylase activity.   Microsomes were isolated
from a pool of livers from either four uninduced
phenobarbital-induced   rats.   Cimetidine
hydrochloride (0.02 - 10 mM) or distilled water
(control) was added *in vitro*.   Results are
expressed as a percent of control activity.
Control   activity   in   nmol/min/mg   protein:
uninduced, 2.22; phenobarbital-induced, 6.24.
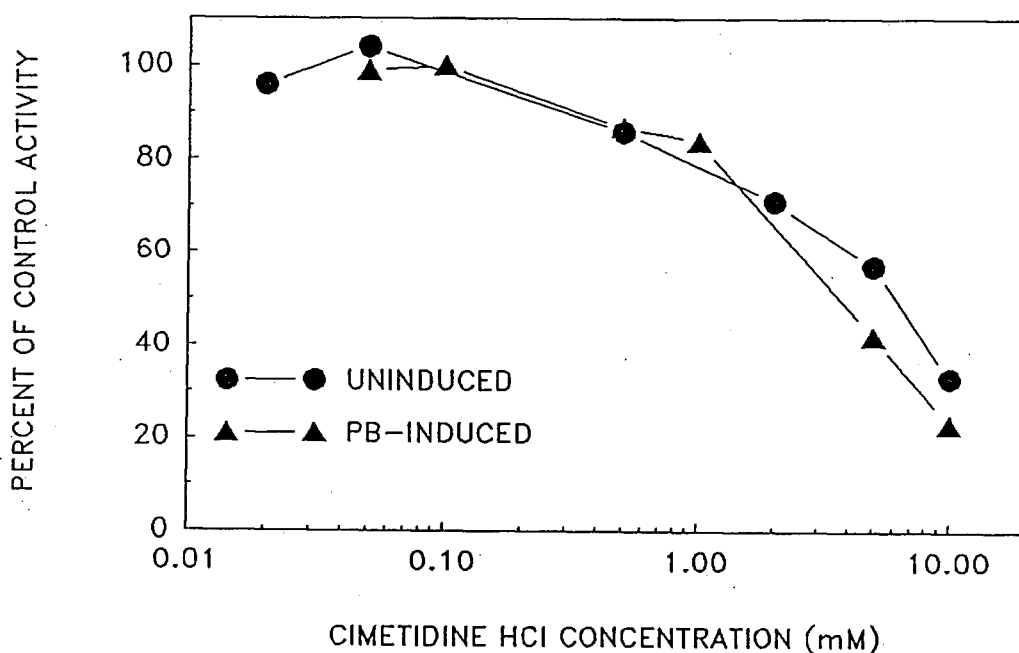
3.1.6.4). Furthermore, the differential effect of *in vivo* cimetidine on testosterone 16α-hydroxylase activity in microsomes from uninduced and phenobarbital-induced rats (Section 3.1.6.4) was not observed with *in vitro* cimetidine.

3.3.8 Testosterone 16β-Hydroxylase Activity

In vitro cimetidine inhibited testosterone 16β-hydroxylase activity in microsomes from phenobarbital-induced rats (Figure 37). The IC_{50} was 3.3 mM (Table 19). This indicates that *in vitro* cimetidine inhibits cytochromes P450IIB1/2 and is in contrast to the apparent lack of inhibition of testosterone 16β-hydroxylase activity in microsomes from phenobarbital-induced rats by *in vivo* cimetidine (Section 3.1.6.5).

3.3.9 Androstenedione Formation

In vitro cimetidine inhibited androstenedione formation in microsomes from uninduced rats (Figure 38). The IC_{50} value was greater than 5 mM (Table 19). This is consistent with the inhibition of androstenedione formation in microsomes from uninduced rats by *in vivo* cimetidine (Section 3.1.6.6).

In vitro cimetidine also inhibited androstenedione formation in microsomes from phenobarbital-induced rats (Figure 38) and the IC_{50} value was 5.5 mM (Table 19). This is in contrast to the apparent lack of inhibition of
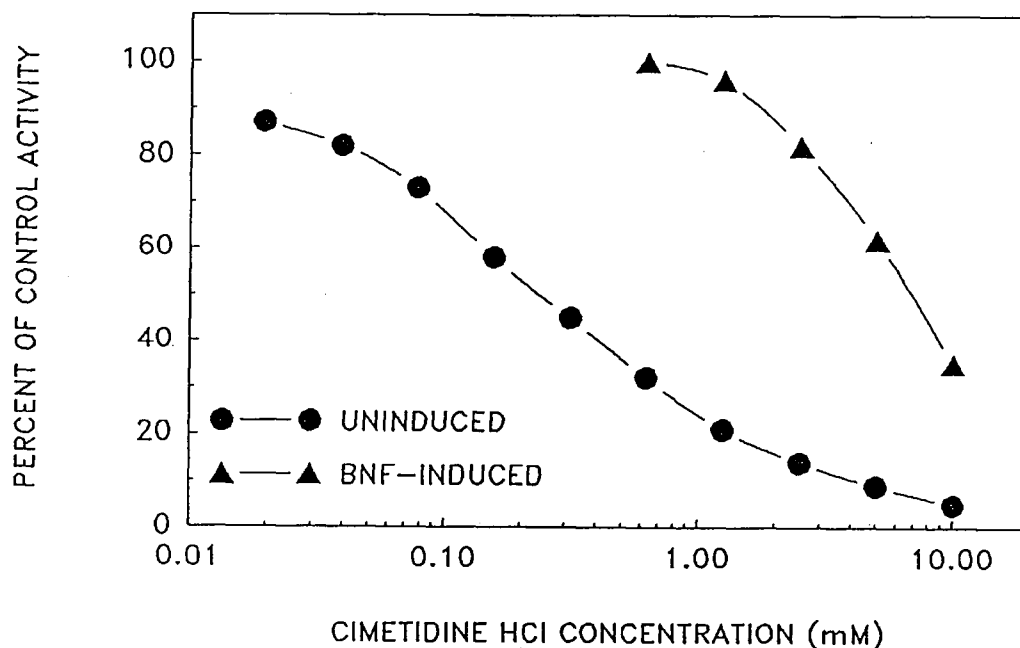
Figure 37 Effect of *in vitro* cimetidine on testosterone 16β-hydroxylase activity. Microsomes were isolated from a pool of livers from four phenobarbital-induced rats. Cimetidine hydrochloride (0.02 - 10 mM) or distilled water (control) was added *in vitro*. Results are expressed as a percent of control activity. Control activity in nmol/min/mg protein: 3.74.
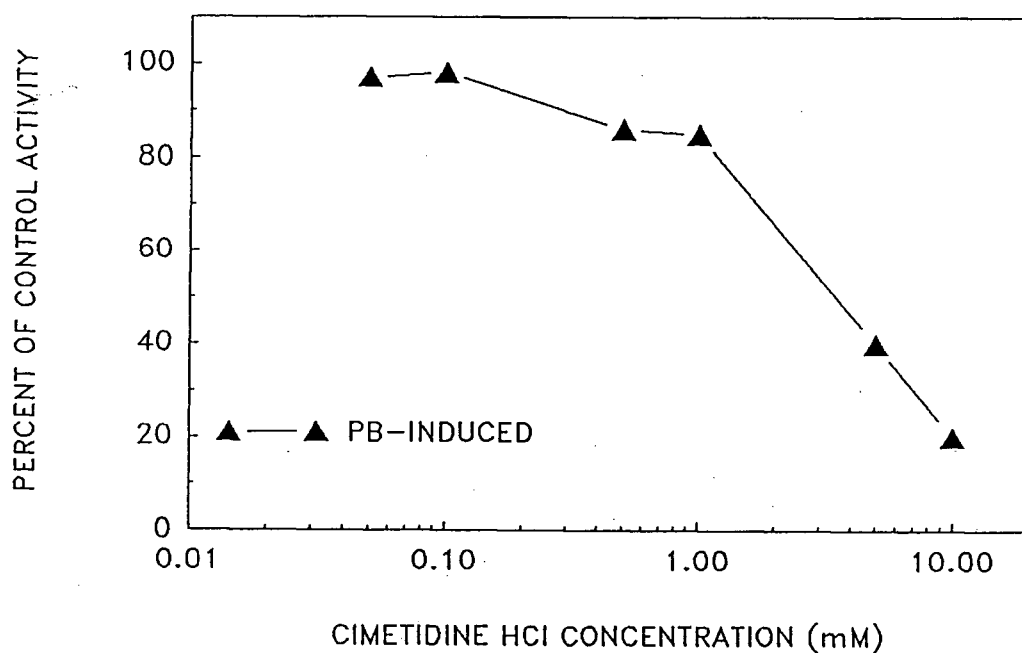
Figure 38 Effect of *in vitro* cimetidine on androstenedione formation. Microsomes were isolated from a pool of livers from either four uninduced or phenobarbital-induced rats. Cimetidine hydrochloride (0.02 - 10 mM) or distilled water (control) was added *in vitro*. Results are expressed as a percent of control activity. Control activity in nmol/min/mg protein: uninduced, 1.34; phenobarbital-induced, 5.23.
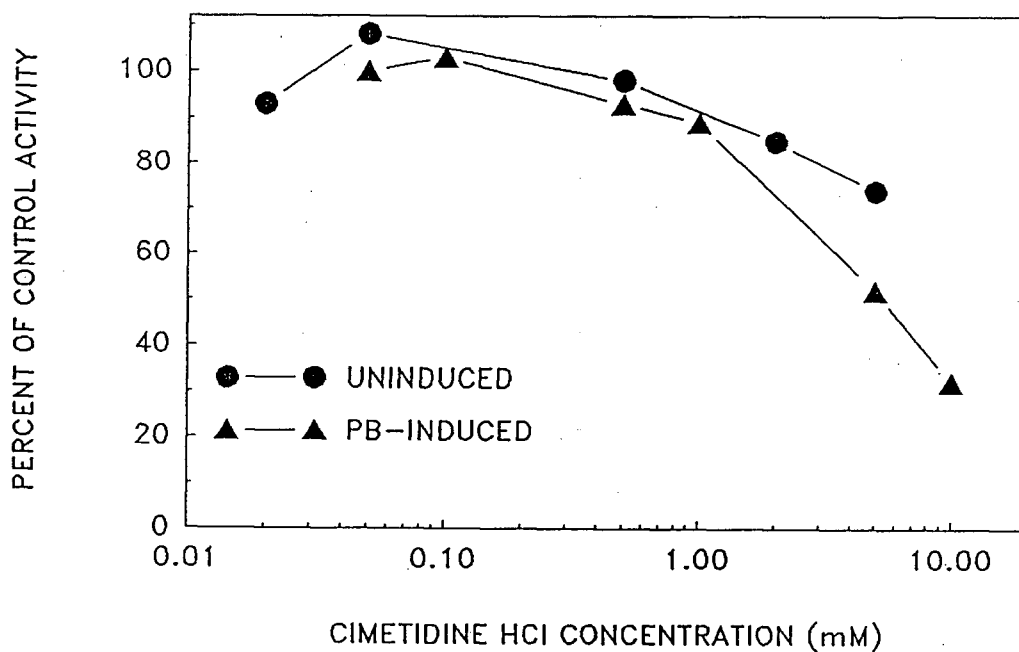
androstenedione formation in microsomes from phenobarbital-induced rats by *in vivo* cimetidine (Section 3.1.6.6).

As was the case with testosterone 16α-hydroxylase activity, the differential effect of *in vivo* cimetidine on androstenedione formation in the uninduced and phenobarbital-induced groups (Section 3.1.6.6) was not observed with *in vitro* cimetidine.

3.3.10 Enzyme Kinetics of the Inhibition of Testosterone 2α-Hydroxylase Activity by *In Vitro* and *In Vivo* Cimetidine

The results presented in the foregoing sections have provided evidence that cimetidine, whether administered *in vitro* or *in vivo*, inhibits cytochrome P450IIC11. To gain further insight into the inhibitory effect of cimetidine on this enzyme, kinetic experiments were performed to determine the type of inhibition of testosterone hydroxylase activity by cimetidine. Microsomes from uninduced adult male rats were used. Based on Lineweaver-Burk plots of the data, the inhibition of testosterone 2α-hydroxylase activity by *in vitro* cimetidine was competitive (Figure 39), whereas the inhibition of this activity by *in vivo* cimetidine was non-competitive (Figure 40).

Figure 39 Linweaver-Burk plot for the inhibition of testosterone 2α-hydroxylase activity by *in vitro* cimetidine. Microsomes were prepared from a pool of four livers from uninduced rats. Cimetidine hydrochloride (2 mM, 4 mM) or distilled water (control) was added *in vitro*. The enzyme activity (V) was determined at various substrate [S] concentrations. The symbols indicate values of the transformed data and the lines were generated by linear regression analysis.
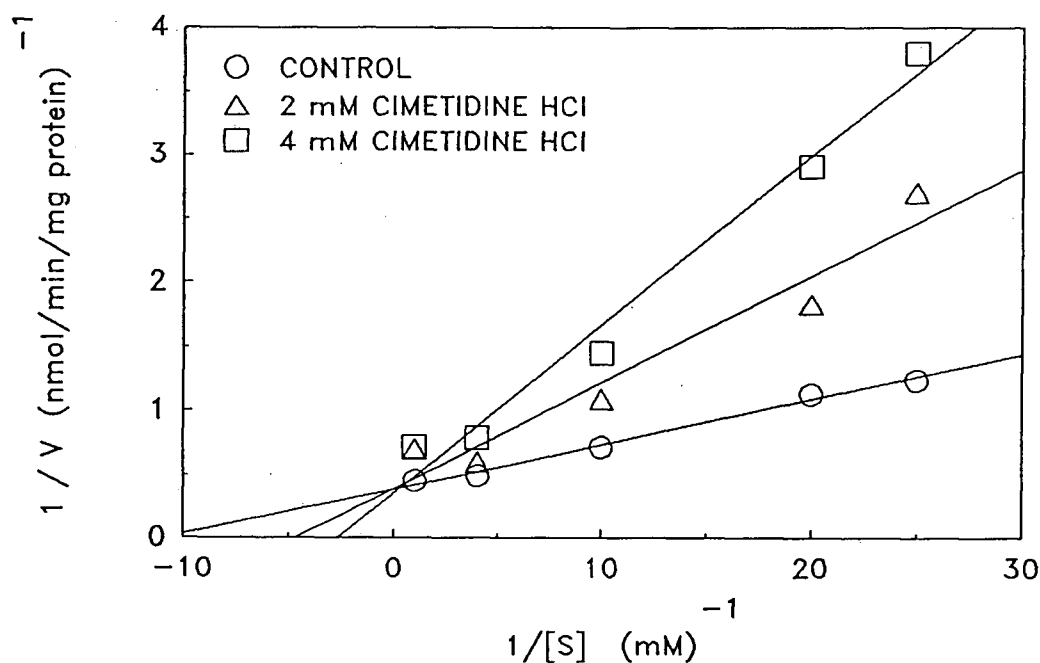
Figure 40 Linweaver–Burk plot for the inhibition of testosterone 2α-hydroxylase activity by *in vivo* cimetidine. Uninduced rats were sacrificed 90 min after a single injection of cimetidine HCl (150 mg/kg) or saline (control). Microsomes were prepared from a pool of four livers in each group. The enzyme activity (V) was determined at various substrate [S] concentrations. The symbols indicate values of the transformed data and the lines were generated by linear regression analysis.
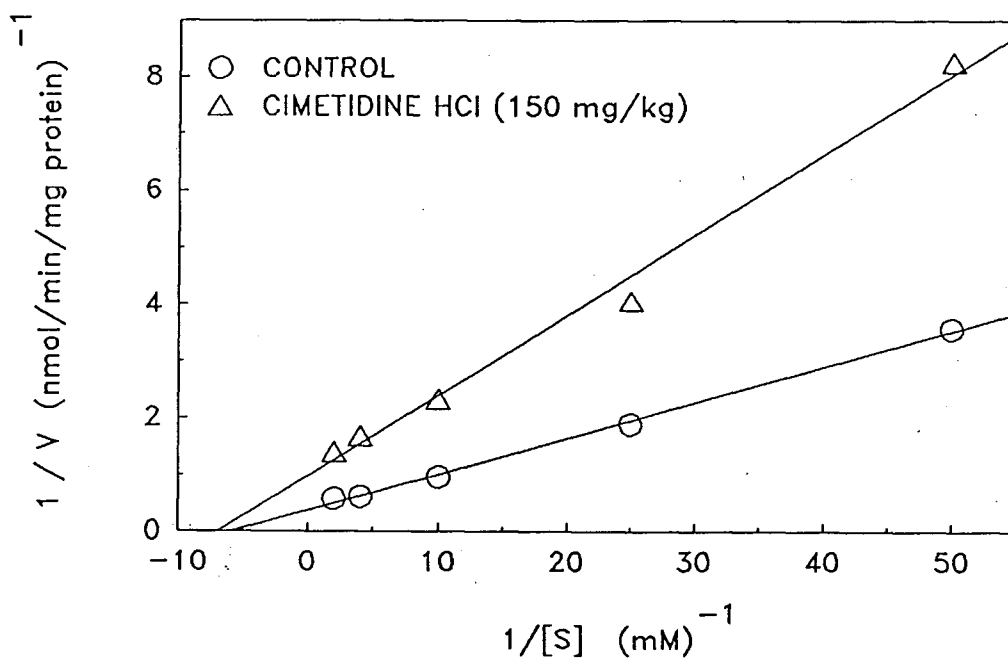
3.4 PREINCUBATION STUDIES WITH *IN VITRO* CIMETIDINE

If cimetidine inhibits cytochrome P-450-mediated hepatic drug metabolism *in vivo* by a non-competitive mechanism, as suggested by the result shown in Figure 40, then this would explain why relatively low concentrations of cimetidine in man and rats can inhibit drug clearance. However, this does not explain why cimetidine is not a more potent inhibitor *in vitro*. Preincubation studies were performed to determine whether cimetidine, at relatively low concentrations, can selectively inhibit microsomal testosterone oxidation in a manner similar to that observed with *in vivo* cimetidine (Section 3.1.6).

3.4.1 Preliminary Experiments

The effect of *in vitro* cimetidine on microsomal testosterone oxidation with the inclusion of a preincubation step in the assay protocol was investigated. In these experiments, microsomes were first preincubated with NADPH and cimetidine. Subsequently, testosterone oxidation was initiated with the addition of the substrate and the reaction was allowed to proceed as described in "Materials and Methods" (Section 2.8.6).

3.4.1.1   Microsomes from Uninduced Rats

The initial experiment was performed to determine whether with a preincubation step, a relatively low concentration of cimetidine can selectively inhibit microsomal testosterone oxidation.   Microsomes were preincubated with cimetidine (0, 0.025, 0.05, 0.1, 0.2 or 0.4 mM) and 1 mM NADPH for 20 min prior to the initiation of testosterone oxidation. As shown in Figure 41, cimetidine resulted in a concentration-dependent inhibition of both testosterone 2α-hydroxylase and testosterone 6β-hydroxylase activities.   However, in microsomes preincubated with 0.05 mM cimetidine and NADPH for 20 min, testosterone 2α-hydroxylase activity was approximately 30% lower compared to those preincubated for the same period of time with NADPH only, whereas testosterone 6β-hydroxylase activity was unaffected by cimetidine (Figure 41).

To determine whether NADPH in the preincubation medium causes any substantial decrease in testosterone oxidation, microsomes were preincubated with 1 mM NADPH for 0 (control) 5, 10, 15 or 20 min prior to the initiation of testosterone oxidation. As shown in Figure 42, the preincubation of microsomes with NADPH (in the absence of cimetidine) for the times indicated resulted in little or no decrease in each of the activities.

An experiment was performed to determine whether the observed inhibition of testosterone 2α-hydroxylase activity

Figure 41 Testosterone 2α- and 6β-hydroxylase activities in microsomes preincubated with NADPH and cimetidine. Livers from four uninduced rats were pooled and microsomes were prepared. Microsomes were preincubated with cimetidine hydrochloride (0.025 - 0.4 mM) or distilled water (control) and NADPH (1 mM) for 20 min. Results are expressed as a percent of control activity. Control activity in nmol/min/mg protein: 2α, 1.67; 6β, 2.01.
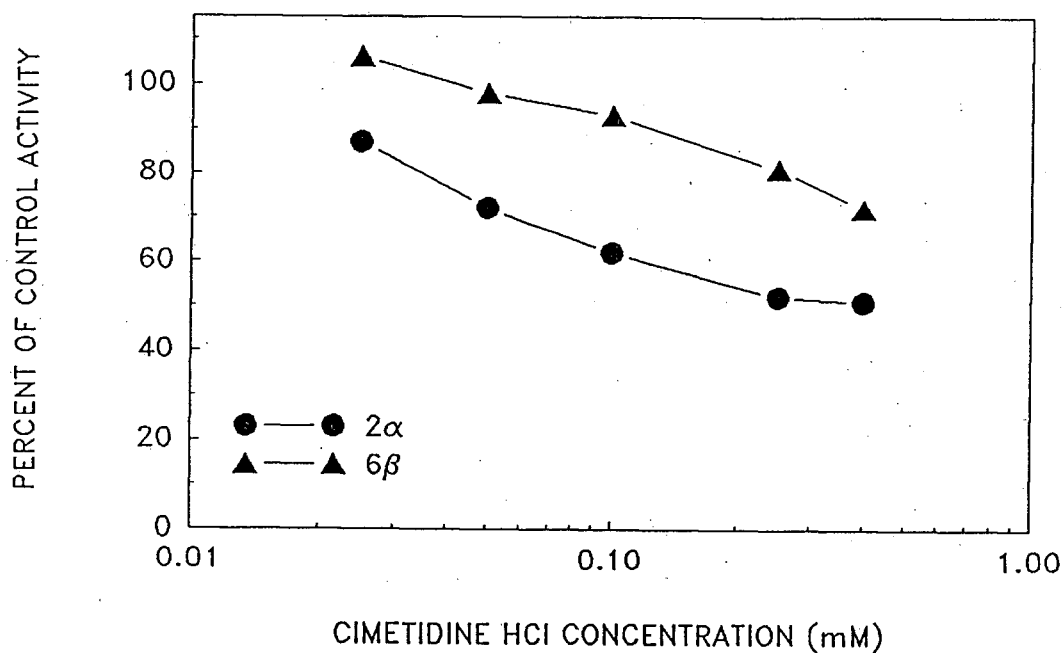
Figure 42 Testosterone oxidation in microsomes preincubated for various times with NADPH in the absence of cimetidine. Livers from four uninduced rats were pooled and microsomes were prepared. Microsomes were preincubated with 1 mM NADPH for 0 (control), 5, 10, 15 or 20 min prior to the initiation of testosterone oxidation. Results are expressed as a percent of control activity. Control activity in nmol/min/mg protein: 2α, 1.72; 2β, 0.05; 6β, 2.11; 7α, 0.25; 16α, 2.20; A (androstenedione), 1.54.
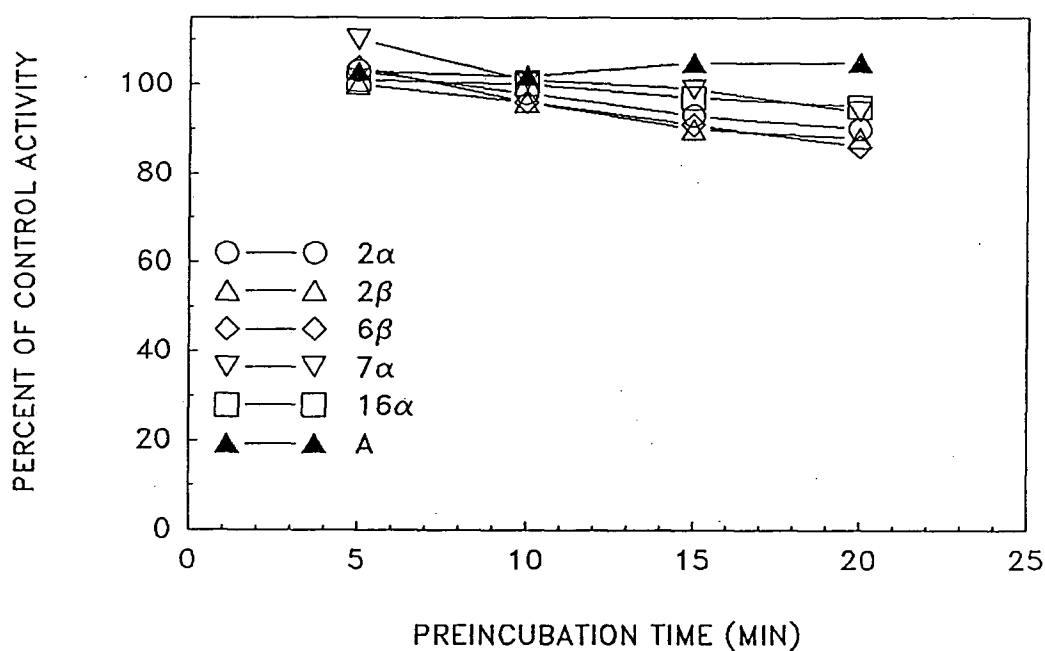
by cimetidine after preincubation is a NADPH-dependent process. Microsomes were preincubated with cimetidine (0, 0.025, 0.1 or 0.4 mM) in the presence or absence of 1 mM NADPH for 20 min prior to the initiation of testosterone oxidation. As shown in Figure 43, NADPH was required in the preincubation medium for the inhibition of testosterone 2α-hydroxylase activity by cimetidine.

A time-course experiment was performed to determine an optimal preincubation period for the selective inhibition of testosterone 2α-hydroxylase activity. Microsomes were preincubated with NADPH and 0.05 mM cimetidine or distilled water (control) for 0, 10, 15, or 20 min prior to the initiation of testosterone oxidation. As shown in Figure 44, near maximal inhibition of testosterone 2α-hydroxylase activity was attained after a 15 min preincubation period at this concentration of cimetidine. No inhibition of testosterone 6β-hydroxylase activity was observed with a preincubation period of up to 20 min (Figure 44).

3.4.1.2   Microsomes from Phenobarbital-Induced Rats

The preincubation step was then used to determine the effect of *in vitro* cimetidine on testosterone oxidation in microsomes from phenobarbital-induced rats. Microsomes were preincubated with 1 mM NADPH and 0, 0.025, 0.05, 0.1 or 0.4 mM cimetidine for 15 min prior to the initiation of

Figure 43 Testosterone 2α-hydroxylase activity in microsomes preincubated with cimetidine in the presence and absence of NADPH. Livers from four uninduced rats were pooled and microsomes were prepared. Microsomes were preincubated with cimetidine hydrochloride (0.025 - 0.4 mM) or distilled water (control) and with or without NADPH (1 mM) for 20 min prior to the initiation of testosterone oxidation. In those cases where NADPH was absent in the preincubation medium, it was added just prior to the start of testosterone oxidation.
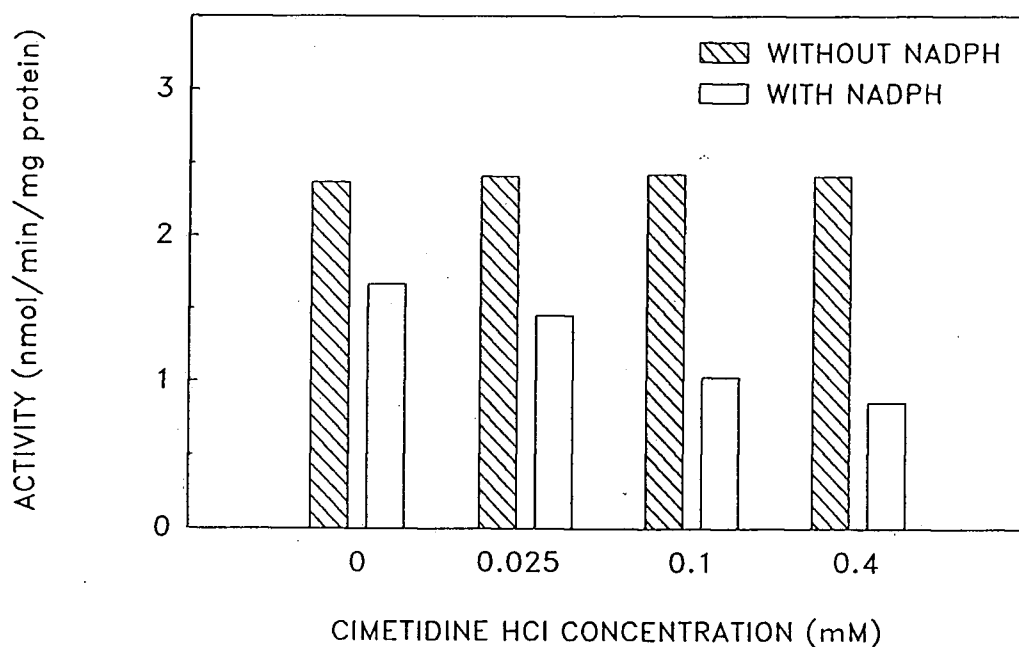
Figure 44 Testosterone 2α- and 6β-hydroxylase activities in microsomes preincubated for various times with 0.05 mM cimetidine and NADPH. Livers from four uninduced rats were pooled and microsomes were prepared. Microsomes were preincubated with cimetidine hydrochloride (0.05 mM) or distilled water (control) and NADPH (1 mM) for 1, 10, 15 or 20 min. Results are expressed as a percent of control activity. Control activity in nmol/min/mg protein for testosterone 2α-hydroxylase: 0 min, 1.72; 10 min, 1.69; 15 min, 1.61; 20 min, 1.59. Control activity in nmol/min/mg protein for testosterone 6β-hydroxylase: 0 min, 2.11; 10 min, 2.03; 15 min, 1.91; 20 min, 1.82.
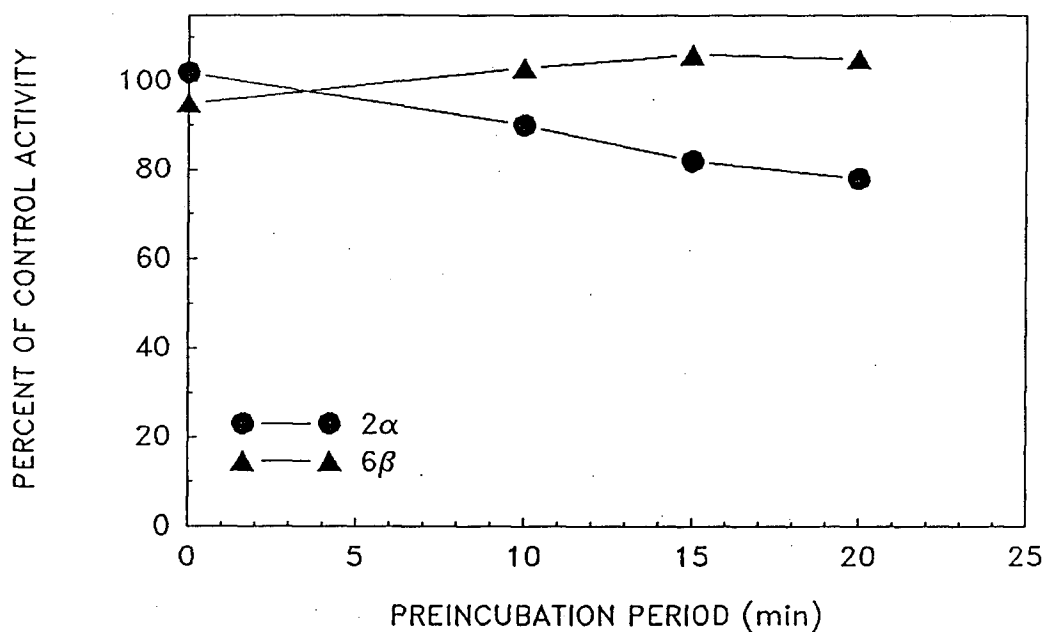
testosterone oxidation. As shown in Figure 45, preincubation of these microsomes with NADPH and 0.025 or 0.05 mM cimetidine for 15 min resulted in the inhibition of testosterone 2α-hydroxylase activity, but not testosterone 6β- or 16β-hydroxylase activity (Figure 45).

3.4.2 Experiments With Individual Microsomal Samples

The preliminary experiments were performed with microsomes prepared from pooled livers. To quantitate the extent of inhibition of testosterone oxidation by *in vitro* cimetidine and determine the selectivity of the inhibition, additional experiments were performed with microsomes prepared from individual livers. The control groups were: 1) no cimetidine and no preincubation; 2) 0.05 mM cimetidine and no preincubation; 3) no cimetidine but the microsomes were preincubated with 1 mM NADPH for 15 min prior to the initiation of testosterone oxidation. In the experimental group, the microsomes were preincubated with 0.05 mM cimetidine and 1 mM NADPH for 15 min prior to the initiation of testosterone oxidation. The results from these experiments are described in the following sections.

3.4.2.1 Testosterone 2α-Hydroxylase Activity

In microsomes from uninduced rats, testosterone 2α-hydroxylase activity was 25% lower (p=0.011) in microsomes preincubated with cimetidine and NADPH than in

Figure 45 Testosterone 2α-, 6β- and 16β-hydroxylase activities in microsomes preincubated for 15 min with cimetidine and NADPH. Livers from four phenobarbital-induced rats were pooled and microsomes were prepared. Microsomes were preincubated with cimetidine hydrochloride (0.025 - 0.2 mM) or distilled water (control) and NADPH (1 mM) for 15 min prior to the initiation of testosterone oxidation. Results are expressed as a percent of control activity. Control activity in nmol/min/mg protein: 2α, 0.78; 6β, 5.97; 16β, 3.62.
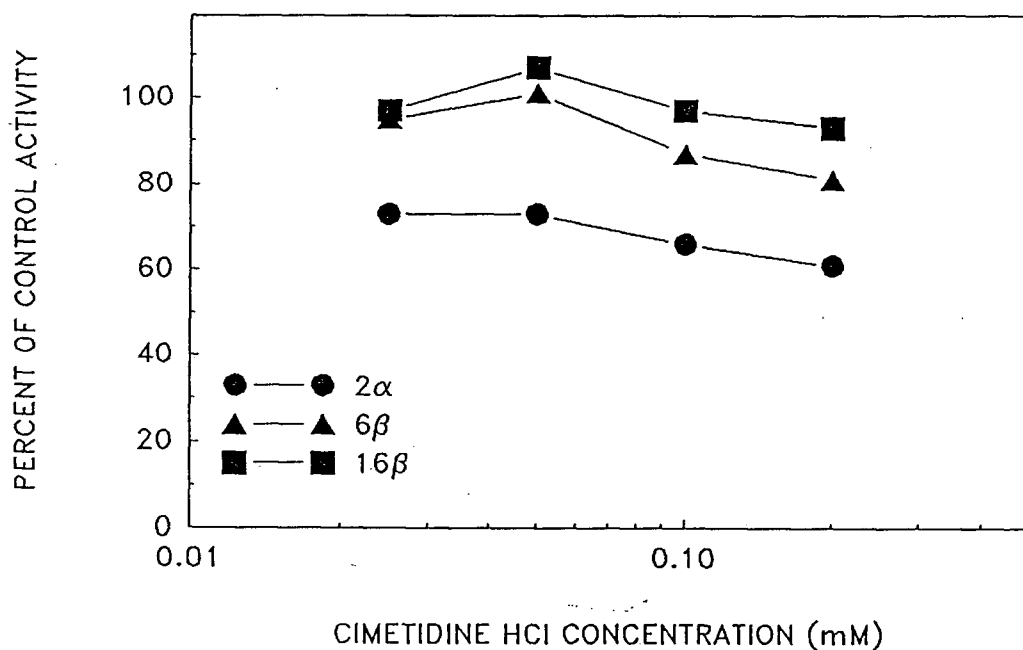
those preincubated with NADPH only (Figure 46A). In microsomes from phenobarbital-induced rats, this activity was 32% lower (p = 0.023) in microsomes preincubated with cimetidine and NADPH than in those preincubated with NADPH only (Figure 46B). These results are consistent with the inhibition of testosterone 2α-hydroxylase activity by *in vivo* cimetidine (Section 3.1.6.1). Without the preincubation step, cimetidine did not inhibit testosterone 2α-hydroxylase activity (Figures 46A and 46B).

3.4.2.2   Testosterone 2β- and 6β-Hydroxylase Activities

With or without the preincubation step, cimetidine did not inhibit the cytochromes P450IIIA1/2-mediated-testosterone 2β- or 6β-hydroxylase activity in microsomes from uninduced or phenobarbital-induced rats (Figures 47A, 47B, 48A and 48B).

3.4.2.3   Testosterone 7α-Hydroxylase Activity

With or without the preincubation step, cimetidine did not inhibit the cytochrome P450IIA1-mediated testosterone 7α-hydroxylase activity in microsomes from uninduced or phenobarbital-induced rats (Figures 49A and 49B).

Figure 46 Effect of preincubation of microsomes with cimetidine and NADPH on testosterone 2α-hydroxylase activity. Microsomes were preincubated with NADPH (1 mM) and cimetidine hydrochloride (0.05 mM) or distilled water for 1 or 15 min prior to the initiation of testosterone oxidation. Results are expressed as the mean ± SEM activity for four individual microsomal samples per group. Panel A: uninduced rats; Panel B: phenobarbital-induced rats. *p < 0.05, compared to the group preincubated with NADPH for the same period of time in the absence of cimetidine.

(A)

(B)
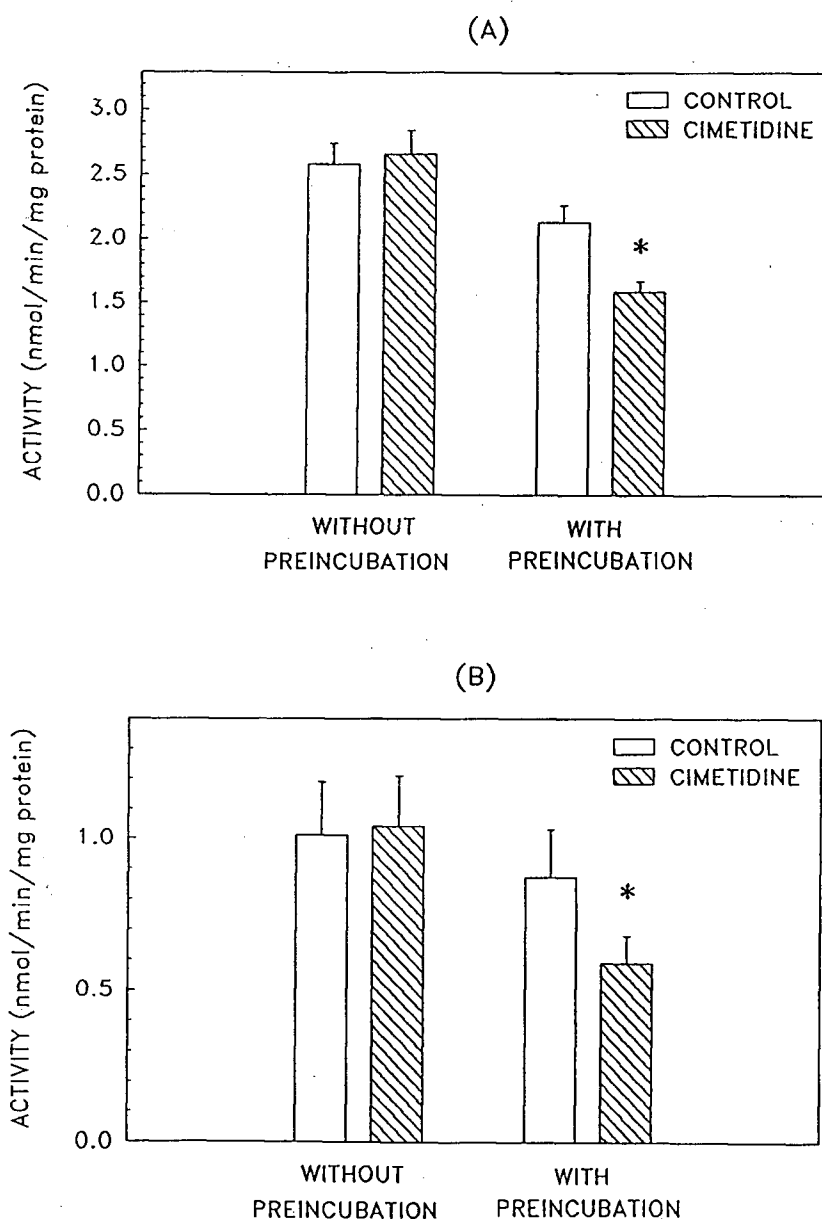
Figure 47 Effect of preincubation of microsomes with cimetidine and NADPH on testosterone 2β-hydroxylase activity. Microsomes were preincubated with NADPH (1 mM) and cimetidine hydrochloride (0.05 mM) or distilled water for 0 or 15 min prior to the initation of testosterone oxidation. Results are expressed as the mean ± SEM activity for four individual microsomal per group. Panel A: uninduced rats; Panel B: phenobarbital-induced rats.

(A)

(B)
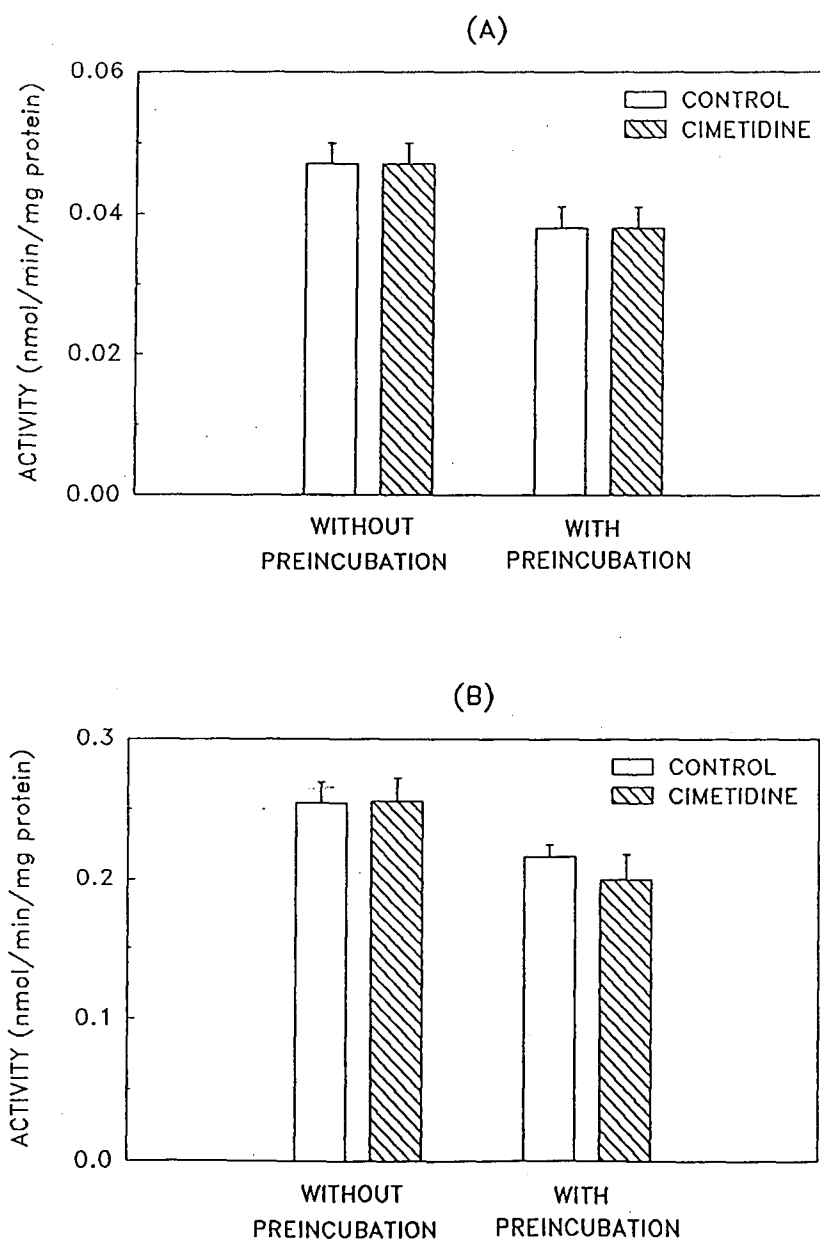
Figure 48 Effect of preincubation of microsomes with cimetidine and NADPH on testosterone 6β-hydroxylase activity. Microsomes were preincubated with NADPH (1 mM) and cimetidine hydrochloride (0.05 mM) or distilled water for 0 or 15 min prior to the initation of testosterone oxidation. Results are expressed as the mean ± SEM activity for four individual microsomal samples per group. Panel A: uninduced rats; Panel B: phenobarbital-induced rats.

(A)

(B)
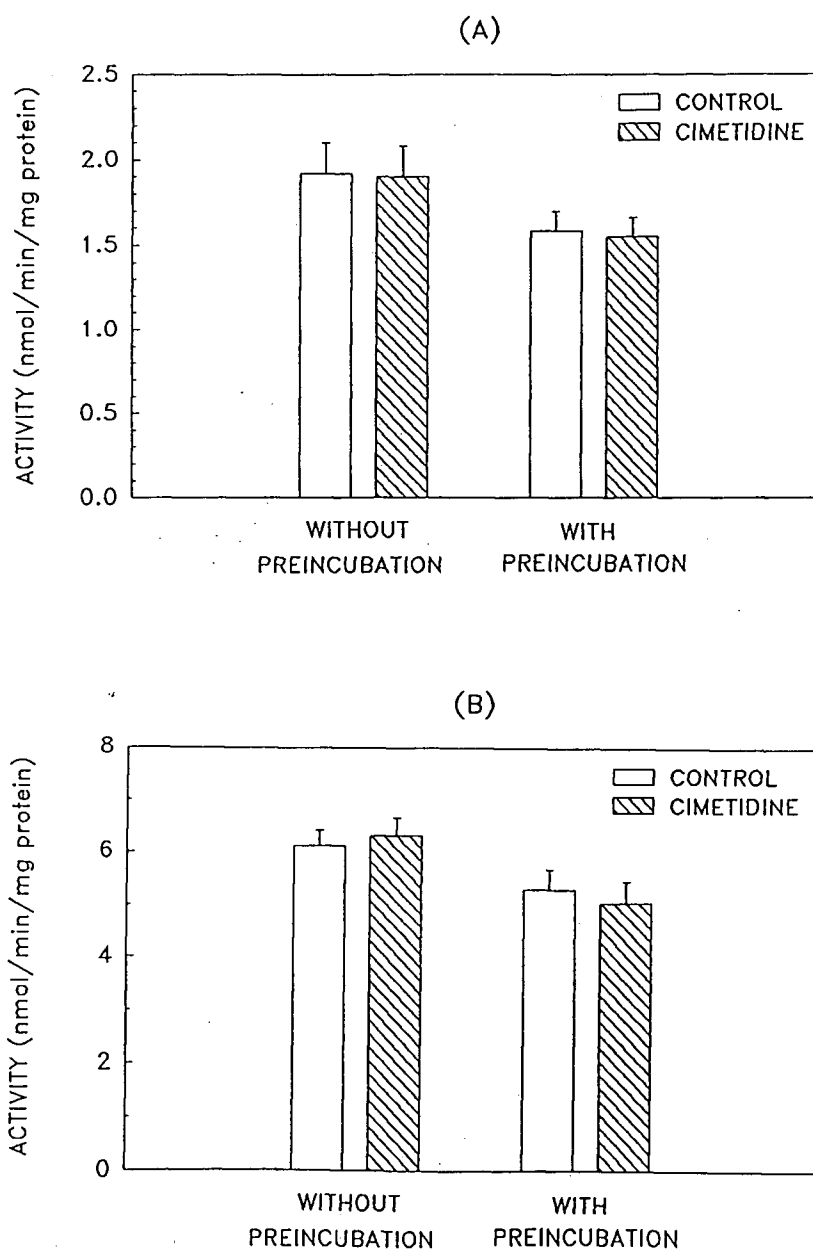
Figure 49 Effect of preincubation of microsomes with cimetidine and NADPH on testosterone 7α-hydroxylase activity. Microsomes were preincubated with NADPH (1 mM) and cimetidine hydrochloride (0.05 mM) or distilled water for 0 or 15 min prior to the initation of testosterone oxidation. Results are expressed as the mean ± SEM activity for four individual microsomal samples per group. Panel A: uninduced rats; and Panel B: phenobarbital-induced rats.

(A)

(B)
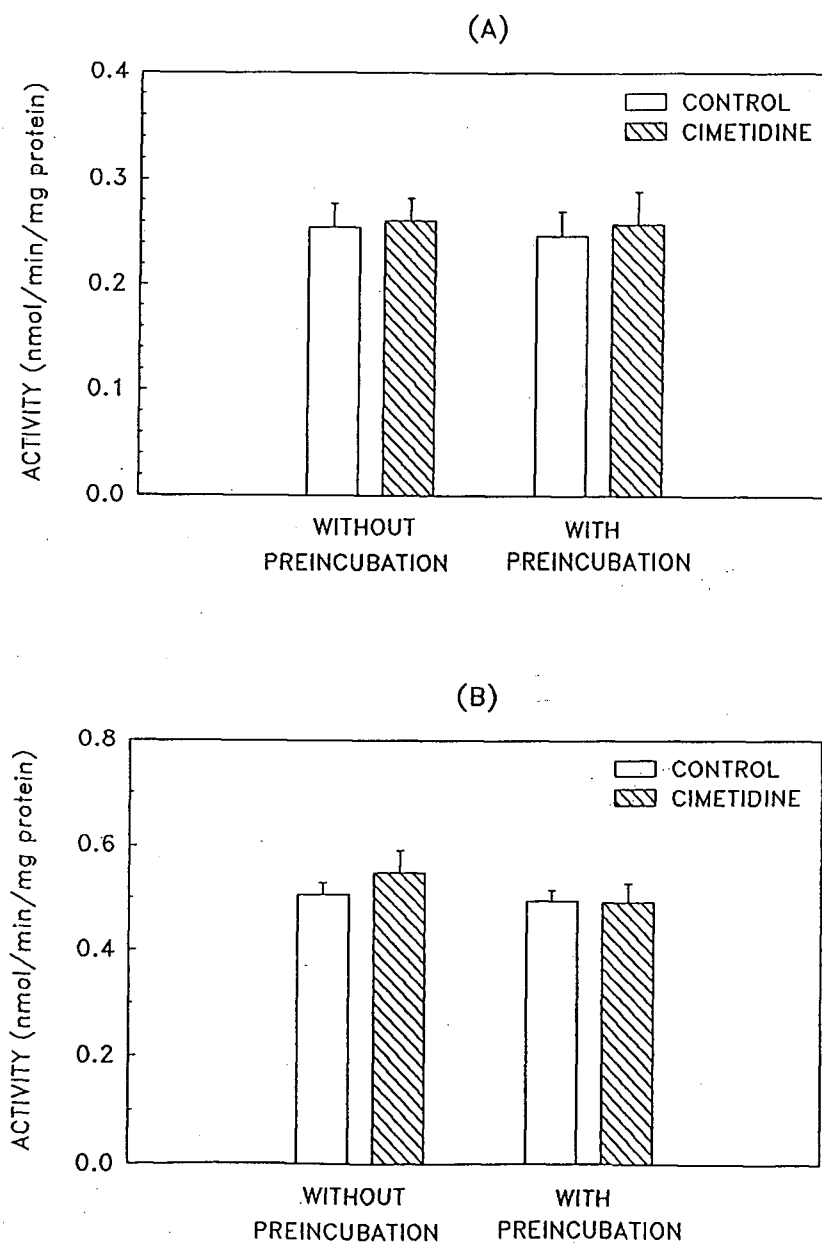
3.4.2.4 Testosterone 16α-Hydroxylase Activity

Testosterone 16α-hydroxylase activity was 27% lower (p=0.009) in microsomes (from uninduced rats) preincubated with cimetidine and NADPH than in those preincubated with NADPH only (Figure 50A). In contrast, with preincubation, cimetidine did not inhibit this activity in microsomes from phenobarbital-induced rats (Figure 50B). This differential effect of *in vitro* cimetidine on testosterone 16α-hydroxylase activity in the uninduced and phenobarbital-induced groups was similar to that observed with *in vivo* cimetidine (Section 3.1.6.4). Without the preincubation step, cimetidine did not inhibit testosterone 16α-hydroxylase activity (Figures 50A and 50B).

3.4.2.5 Testosterone 16β-Hydroxylase Activity

With or without the preincubation step, cimetidine did not inhibit the cytochromes P450IIB1/2-mediated testosterone 16β-hydroxylase activity in microsomes from phenobarbital-induced rats (Figure 51).

3.4.2.6 Androstenedione Formation

With or without the preincubation step, cimetidine did not inhibit androstenedione formation in microsomes from uninduced or phenobarbital-treated rats (Figures 52A and 52B).

Figure 50 Effect of preincubation of microsomes with cimetidine and NADPH on testosterone 16α-hydroxylase activity. Microsomes were preincubated with NADPH (1 mM) and cimetidine hydrochloride (0.05 mM) or distilled water for 0 or 15 min prior to the initation of testosterone oxidation. Results are expressed as the mean ± SEM activity for four individual microsomal samples per group. Panel A: uninduced rats; Panel B: phenobarbital-induced rats. *p < 0.01, compared to the group preincubated with NADPH for the same period of time in the absence of cimetidine.

(A)

(B)
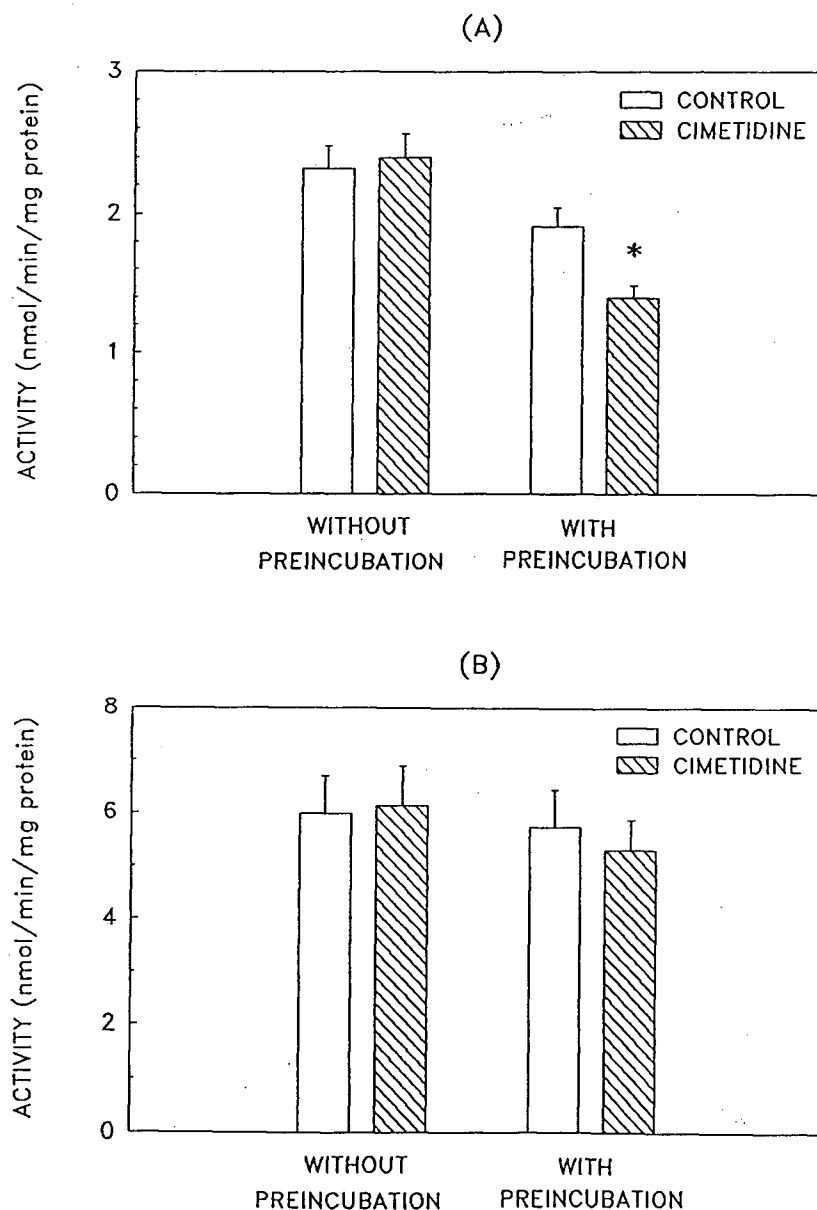
Figure 51 Effect of preincubation of microsomes with cimetidine and NADPH on testosterone 16β-hydroxylase activity. Microsomes (from phenobarbital-induced rats) were preincubated with NADPH (1 mM) and cimetidine hydrochloride (0.05 mM) or distilled water for 0 or 15 min prior to the initation of testosterone oxidation. Results are expressed as the mean ± SEM activity for four individual microsomal samples per group.
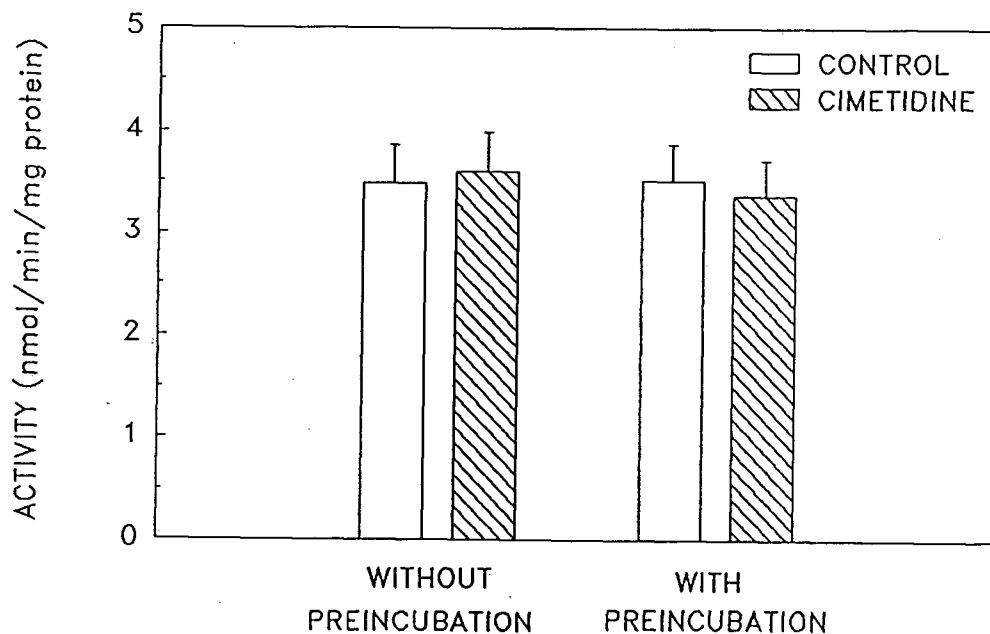
Figure 52 Effect of preincubation of microsomes with cimetidine and NADPH on androstenedione activity. Microsomes were preincubated with NADPH (1 mM) and cimetidine hydrochloride (0.05 mM) or distilled water for 0 or 15 min prior to the initation of testosterone oxidation. Results are expressed as the mean ± SEM activity for four individual microsomal samples per group. Panel A: uninduced rats; Panel B: phenobarbital-induced rats.

(A)

(B)
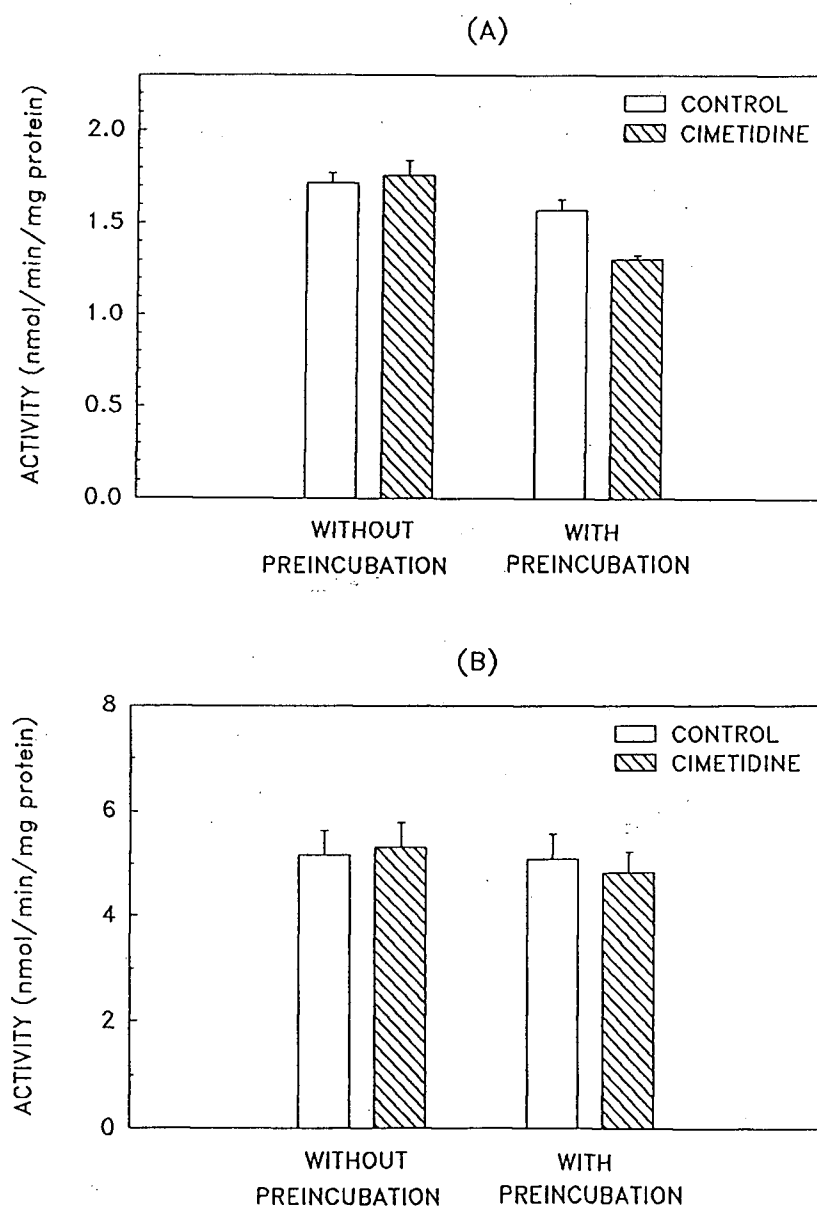
3.5 SUMMARY OF THE EFFECTS OF *IN VIVO* AND *IN VITRO* CIMETIDINE ON MICROSOMAL TESTOSTERONE OXIDATION

In microsomes from uninduced adult male rats, *in vivo* cimetidine inhibited testosterone 2α- and 16α-hydroxylase activities and androstenedione formation, but not testosterone 2β, 6β- or 7α-hydroxylase activity (Figure 53A). *In vitro* cimetidine (5 mM) inhibited all of these activities, except for testosterone 7α-hydroxylase activity (Figure 53B). With the inclusion of the preincubation step, a low concentration (0.05 mM) of *in vitro* cimetidine inhibited only testosterone 2α- and 16α-hydroxylase activities (Figure 53C).

In microsomes from phenobarbital-induced adult male rats, *in vivo* cimetidine inhibited testosterone 2α-hydroxylase activity, but not testosterone 2β-, 6β, 7α-, 16α- or 16β-hydroxylase activity or androstenedione formation (Figure 54A). *In vitro* cimetidine (5 mM) inhibited all of these activities, except for testosterone 7α-hydroxylase activity (Figure 54B). With the inclusion of the preincubation step, *in vitro* cimetidine (0.05 mM) inhibited only testosterone 2α-hydroxylase activity (Figure 54C).

Figure 53 Summary of the effects of *in vivo* and *in vitro* cimetidine on testosterone oxidation in microsomes from uninduced rats. (A) *In vivo* cimetidine; (B) *in vitro* cimetidine (5 mM); (C) *in vitro* cimetidine (0.05 mM) with preincubation. Results are based on the data from Figures 18, 32-38 and 46-52. #p < 0.001, 'p < 0.01, *p < 0.05 compared to the respective control group. In Panel B, statistical analyses were not performed since the microsomes were prepared from a pool of livers.

(A)

(B)

(C)
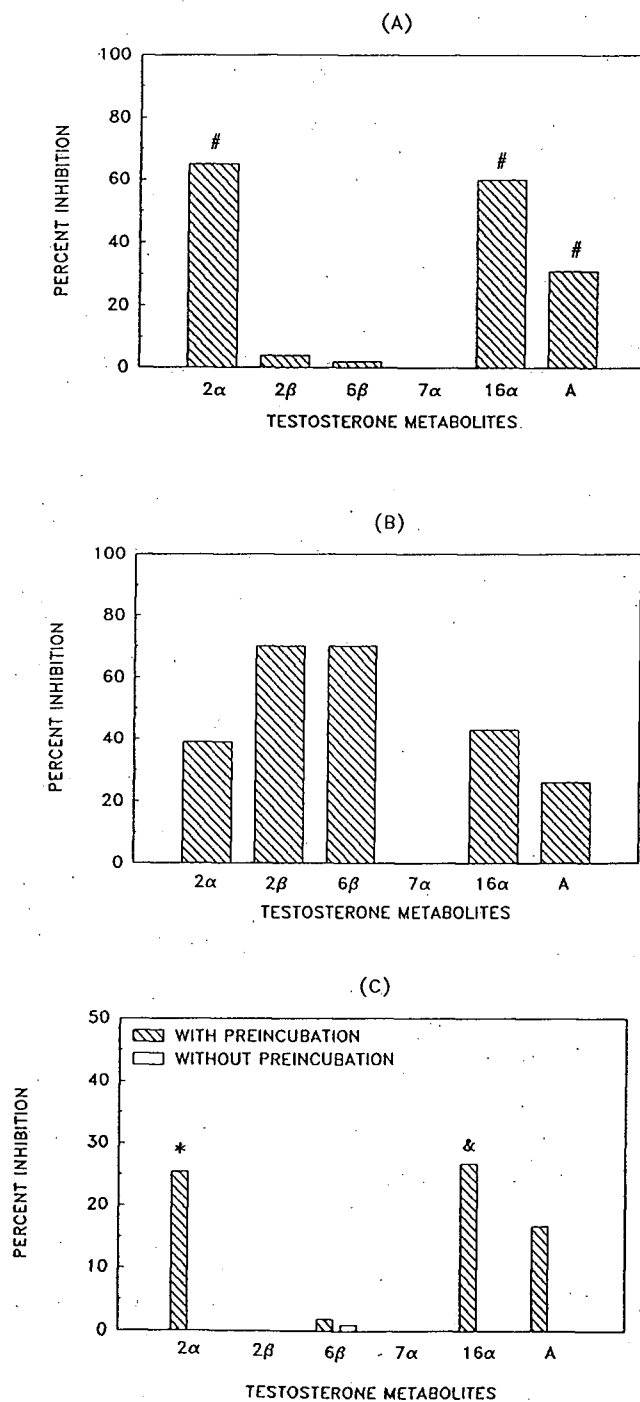
Figure 54 Summary of the effects of *in vivo* and *in vitro* cimetidine on testosterone oxidation in microsomes from phenobarbital-induced rats. (A) *In vivo* cimetidine; (B) *in vitro* cimetidine (5 mM); (C) *in vitro* cimetidine (0.05 mM) with preincubation. Results are based on the data from Figures 18, 32-38 and 46-52. $^{\#}$p < 0.001, *p < 0.005 compared to the respective control group. In Panel B, statistical analyses were not performed since the microsomes were prepared from a pool of livers.
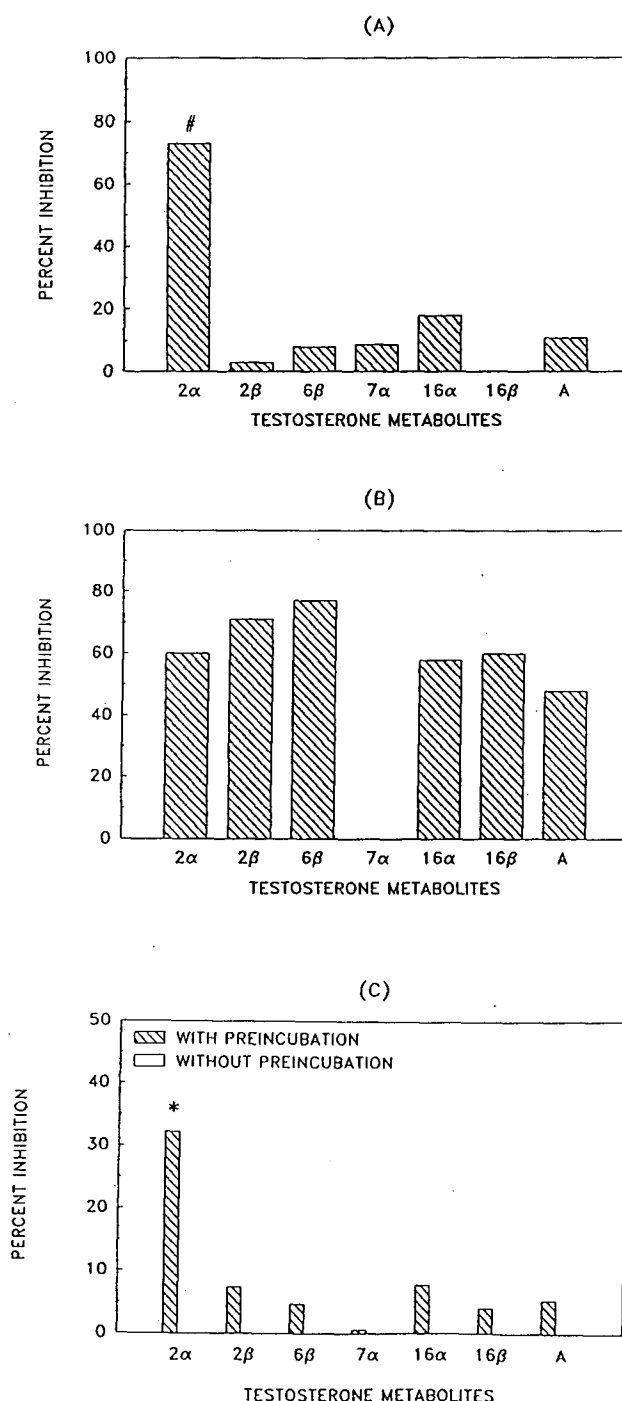
DISCUSSION

4.1 DIFFERENTIAL INHIBITION OF CYTOCHROME P-450-MEDIATED ENZYME ACTIVITIES BY *IN VIVO* CIMETIDINE

4.1.1 Inhibition of Cytochrome P450IIC11 by *In Vivo* Cimetidine

Observations from human and animal studies have led to the perception that cimetidine is a general inhibitor of hepatic cytochrome P-450 enzymes (Reilly *et al.*, 1988; Leclercq *et al.*, 1989). However, there is substantial, but indirect, evidence that cimetidine may differentially inhibit hepatic cytochrome P-450 enzymes. An initial observation from the present investigation was that *in vivo* cimetidine inhibited aminopyrine N-demethylase activity to a greater extent in microsomes from uninduced than in those from induced adult male rats (Figures 8 and 9). Slusher *et al.* (1987) reported that anti-cytochrome P450IA1/2 and anti-cytochrome P450IIB1/2 antibodies partially inhibited the formation of 4-aminoantipyrine, one of the N-demethylated metabolites of aminopyrine, in microsomes from adult male rats induced with phenobarbital or 3-methylcholanthrene, but not in microsomes from uninduced adult male rats. This indicates that different cytochrome P-450 enzymes are responsible for aminopyrine N-demethylase activity in

microsomes from uninduced and induced rats. The results shown in Figures 8 and 9, in conjunction with the observations of Slusher *et al.* (1987), suggested that at least one of the cytochrome P-450 enzymes present in uninduced rats was more susceptible to inhibition by *in vivo* cimetidine than those present in induced rats. Therefore, further studies were performed to test the hypothesis that *in vivo* cimetidine differentially inhibits hepatic cytochrome P-450 enzymes in adult male rats.

Cytochrome P450IIC11 is a major hepatic cytochrome P-450 enzyme in uninduced adult male rats (Guengerich *et al.*, 1982a; Waxman *et al.*, 1985). The results from the present investigation provide the first evidence that *in vivo* cimetidine inhibits cytochrome P450IIC11. The evidence for this is derived from the cases in which an enzyme activity has been found to be due entirely to cytochrome P450IIC11. *In vivo* cimetidine inhibited testosterone 2α- and 16α-hydroxylase activities in microsomes from uninduced rats (Figures 10, 14 and 18). Consistent with published results (Waxman, 1984; Waxman *et al.*, 1987), the monospecific anti-cytochrome P450IIC11 completely inhibited testosterone 2α-hydroxylase activity (Figure 22) and inhibited testosterone 16α-hydroxylase activity by 95% (Figure 23) in microsomes from uninduced adult male rats. Since *in vivo* cimetidine inhibited both of these activities by more than 60% in microsomes from uninduced rats (Figures

10, 14 and 18), it is apparent that cytochrome P450IIC11 is subject to inhibition by this drug.

The results of antibody inhibition experiments showed that the testosterone 2α-hydroxylase activity was also entirely due to cytochrome P450IIC11 in microsomes from phenobarbital-induced and dexamethasone-induced rats (Figure 22). This activity was also inhibited by *in vivo* cimetidine in microsomes from these two pretreatment groups (Figure 10 and 18). In uninduced rats, the antibody was also found to inhibit pentoxyresorufin 0-dealkylase activity by more than 90% (Figure 20), indicating that cytochrome P450IIC11 catalyzed this activity in the absence of induction. *In vivo* cimetidine inhibited pentoxyresorufin 0-dealkylase activity by 38% in microsomes from uninduced rats (Figure 5). Recently, Nakajima *et al.* (1990) reported that an antibody to cytochrome P450IIC11, with cross-reactivity to cytochrome P450IIC6, completely inhibited pentoxyresorufin 0-dealkylase activity in microsomes from uninduced rats.

In the present investigation, in each case where cytochrome P450IIC11 was observed to be the major or sole contributor to an enzyme activity, that activity was inhibited by *in vivo* cimetidine.

4.1.2 Lack of Inhibition of Cytochromes P450IIB1/2, Cytochromes P450IIIA1/2 and Cytochrome P450IIA1 by *In Vivo* Cimetidine

Cytochromes P450IIB1/2 are the major cytochrome P-450 enzymes inducible by phenobarbital (Guengerich *et al.*, 1982a; Thomas *et al.*, 1983; Waxman *et al.*, 1985). Testosterone 16β-hydroxylase and pentoxyresorufin 0-dealkylase activities in microsomes from phenobarbital-induced rats are frequently used as markers for cytochromes P450IIB1/2 (Lubet *et al.*, 1985; Reik *et al.*, 1985; Waxman *et al.*, 1985, 1987; Dutton and Parkinson, 1989). *In vivo* cimetidine did not inhibit these two activities in microsomes from phenobarbital-induced rats (Figures 5 and 15). If cimetidine is a competitive inhibitor of these two activities, then the observed lack of inhibition may have been the result of a relatively high substrate concentration. However, even at lower substrate concentrations, *in vivo* cimetidine did not affect testosterone 16β-hydroxylase activity in microsomes from phenobarbital-induced rats (Figure 16). Alternatively, the apparent lack of inhibition of these two activities by cimetidine could be explained by an increase in the clearance of cimetidine from the animals due to the induction of cytochromes P-450 by the phenobarbital pretreatment. This would require, as well, that cimetidine is the active inhibitor and that the inhibition is competitive. However, in the same microsomes in which there

was lack of inhibition of testosterone 16β-hydroxylase and pentoxyresorufin 0-dealkylase activities, *in vivo* cimetidine inhibited testosterone 2α-hydroxylase activity by 73% (Figures 10 and 18), indicating that the active form of the inhibitor was present in the microsomes. In fact, the extent of inhibition of testosterone 2α-hydroxylase activity by cimetidine in microsomes from phenobarbital-induced rats was similar to that observed in microsomes from uninduced rats (Figure 18).

The apparent lack of inhibition of testosterone 16α-hydroxylase activity by *in vivo* cimetidine in microsomes from phenobarbital-induced rats (Figure 14) is also consistent with the suggestion that *in vivo* cimetidine does not inhibit cytochromes P450IIB1/2. It has been shown in immunoinhibition studies that cytochromes P450IIB1/2 account for the majority of the testosterone 16α-hydroxylase activity in microsomes from phenobarbital-induced rats (Thomas *et al.*, 1981; Reik *et al.*, 1985; Waxman *et al.*, 1987). Therefore, the results from the present investigation indicate that *in vivo* cimetidine administration to adult male rats apparently does not inhibit cytochromes P450IIB1/2.

Cytochromes P450IIIA1/2 are the major cytochrome P-450 enzymes inducible by dexamethasone (Heuman *et al.*, 1982). Testosterone 2β- and 6β-hydroxylase activities are markers for cytochromes P450IIIA1/2 in microsomes from uninduced,

phenobarbital-induced and dexamethasone-induced rats (Halvorson *et al.*, 1990). Erythromycin N-demethylase activity is also used as a marker for cytochromes P450IIIA1/2 in microsomes from dexamethasone-induced rats (Wrighton *et al.*, 1985a). *In vivo* cimetidine did not affect testosterone 2β- or 6β-hydroxylase activity in microsomes from uninduced, phenobarbital- or dexamethasone-induced rats (Figures 11, 12 and 18). As well, *in vivo* cimetidine did not inhibit erythromycin N-demethylase activity in microsomes from dexamethasone-induced rats (Figures 6 and 8). However, in the same microsomes from dexamethasone-induced rats, *in vivo* cimetidine did inhibit testosterone 2α-hydroxylase activity (Figures 10 and 18), and this activity was mediated by cytochrome P450IIC11 (Figure 22). It appears that under the experimental conditions of the present investigation, *in vivo* cimetidine does not inhibit cytochromes P450IIIA1/2. In a preliminary report, Cooper *et al.* (1990) claimed that both cytochrome P450IIIA1 and cytochrome P450IIIA2 are present in livers of dexamethasone-treated adult male rats, whereas cytochrome P450IIIA2, but not cytochrome P450IIIA1, is expressed in livers of uninduced adult male rats.

Cytochrome P450IIA1 is a female-predominant cytochrome P-450 enzyme (Waxman *et al.*, 1989) and is a minor constituent in livers of uninduced adult male rats (Guengerich *et al.*, 1982a; Waxman *et al.*, 1985). Testosterone 7α-hydroxylase activity is used as a marker for

cytochrome P450IIA1 in microsomes from uninduced, phenobarbital-induced and dexamethasone-induced adult male rats (Levin *et al.*, 1987; Waxman *et al.*, 1988b; Arlotto and Parkinson, 1989). Since *in vivo* cimetidine did not inhibit this activity in microsomes from these groups of adult male rats (Figures 13 and 18), it appears that, under the experimental conditions of the present investigation, *in vivo* cimetidine does not inhibit cytochrome P450IIA1.

4.1.3 Indirect Evidence for the Inhibition of Other Cytochrome P-450 Enzymes by *In Vivo* Cimetidine

There is evidence from the present investigation that other cytochrome P-450 enzymes in uninduced adult male rats, in addition to cytochrome P450IIC11, are also inhibited by *in vivo* cimetidine.

In vivo cimetidine inhibited erythromycin N-demethylase activity by 40% in microsomes from uninduced rats (Figures 6 and 8), yet the anti-cytochrome P450IIC11 antibody had little or no effect on this activity in these microsomes (Figure 21). Therefore, the inhibition of erythromycin N-demethylase activity by cimetidine in uninduced rats is likely to be due to an enzyme(s) other than cytochrome P450IIC11. Based on the observation of a biphasic Eadie-Hofstee plot, it has been suggested that at least two cytochrome P-450 enzymes are responsible for erythromycin N-demethylase activity in microsomes from uninduced adult male rats (Chang *et al.*, 1990), but it has not been

demonstrated in an immunoinhibition experiment which cytochrome P-450 enzyme(s) contribute(s) to this activity in microsomes from this group of rats. However, the enzyme that catalyzes erythromycin N-demethylase in the uninduced state is unlikely to be cytochrome P450IIA1, cytochromes P450IIB1/2 or cytochromes P450IIIA1/2 since *in vivo* cimetidine did not inhibit the activities that are specific for these enzymes.

Aminopyrine N-demethylase activity in microsomes from uninduced rats was inhibited 62% by *in vivo* cimetidine (Figures 4 and 8). Since cytochrome P450IIC11 accounted for only approximately 35% of the aminopyrine N-demethylase activity in these microsomes (Figure 19), it is possible that *in vivo* cimetidine inhibits another cytochrome P-450 enzyme(s) which contribute(s) to this activity in uninduced rats.

4.1.4 Effect of *In Vivo* Cimetidine on Cytochrome P450IA1 and Cytochrome P450IA2

Cytochrome P450IA1 is the major cytochrome P-450 enzyme inducible by β-naphthoflavone and 3-methylcholanthrene (Guengerich *et al.*, 1982a; Thomas *et al.*, 1983; Waxman *et al.*, 1985). Purified cytochrome P450IA1 catalyzes ethoxyresorufin O-deethylation (Goldstein *et al.*, 1982; Guengerich *et al.*, 1982a; Astrom and DePierre, 1985). Kelley *et al.* (1987) reported that an anti-cytochrome P450IA1 antibody inhibited ethoxyresorufin O-deethylase

activity in microsomes from 3-methylcholanthrene-induced rats by 82% and an anti-cytochrome P450IA2 antibody inhibited this activity in microsomes from the same group of rats by only 27%. Recently, Nakajima *et al.* (1990) reported that an antibody to cytochrome P450IA1, with cross-reactivity to cytochrome P450IA2, inhibited ethoxyresorufin 0-deethylase activity in microsomes from 3-methylcholanthrene-induced rats by 79%. However, it has not been shown in an immunoinhibition experiment whether cytochrome P450IA1 is the major enzyme responsible for ethoxyresorufin 0-deethylase activity in microsomes from β-naphthoflavone-induced rats. In the present study, *in vivo* cimetidine did not inhibit this activity in microsomes from β-naphthoflavone-induced rats (Figures 7 and 9). As an internal control, aminopyrine N-demethylase activity was determined in microsomes from β-naphthoflavone-induced rats. As shown in Figures 4 and 9, *in vivo* cimetidine did inhibit aminopyrine N-demethylase activity by 28% in these microsomes (Figures 4 and 9), indicating that the inhibitory action of cimetidine was present after β-naphthoflavone pretreatment. If cytochrome P450IA1 is the enzyme responsible for ethoxyresorufin 0-deethylase activity in microsomes from β-naphthoflavone-induced rats, then it would appear that, under the experimental conditions in the present investigation, *in vivo* cimetidine does not inhibit cytochrome P450IA1.

Cytochrome P450IA2 is a major cytochrome P-450 enzyme inducible by isosafrole and is present in low levels in uninduced rats (Guengerich *et al.*, 1982a; Thomas *et al.*, 1983; Waxman *et al.*, 1985). Kelley *et al.* (1987) reported that an anti-cytochrome P450IA2 antibody inhibited ethoxyresorufin 0-deethylase activity by 78% in microsomes from uninduced rats. In the present study, this activity was used as a marker for cytochrome P450IA2 in microsomes from uninduced rats. It was found that *in vivo* cimetidine inhibited ethoxyresorufin 0-deethylase activity by 84% in microsomes from this group of rats (Figures 7 and 9). Since the completion of this experiment, Nakajima *et al.* (1990) reported that an antibody to cytochrome P450IIC11, with cross-reactivity to cytochrome P450IIC6, inhibited ethoxyresorufin 0-deethylase activity by 74% in microsomes from uninduced rats. It is therefore uncertain whether ethoxyresorufin 0-deethylase activity is a reliable marker for cytochrome P450IA2 in microsomes from uninduced rats. It is possible that both cytochrome P450IA2 and cytochrome P450IIC11 catalyze ethoxyresorufin 0-deethylation in microsomes from uninduced rats. Future experiments should be performed to clarify the enzyme-specificity of ethoxyresorufin 0-deethylase activity in microsomes from uninduced rats and the effect of *in vivo* cimetidine on cytochrome P450IA2.

4.1.5 Effect of Pretreatment on Inhibition of Cytochrome P-450 by Cimetidine

In some cases, the extent of inhibition of enzyme activities by *in vivo* cimetidine depended on prior treatment with a cytochrome P-450 inducer. This appeared to be due to the increasing contribution to such activities by inducible enzymes which were not subject to inhibition by cimetidine. *In vivo* cimetidine inhibited testosterone 16α-hydroxylase activity in microsomes from uninduced rats, but did not affect this activity in microsomes from phenobarbital-induced rats (Figure 14). The anti-cytochrome P450IIC11 antibody inhibited testosterone 16α-hydroxylase activity in microsomes from uninduced rats, but had little or no effect on this activity in microsomes from phenobarbital-induced rats (Figure 23), consistent with published data (Waxman, 1984; Waxman *et al.*, 1987). Cytochromes P450IIB1/2 are the major contributors to testosterone 16α-hydroxylase activity in hepatic microsomes from phenobarbital-induced rats (Thomas *et al.* 1981; Reik *et al.*, 1985; Waxman *et al.*, 1987), but does not contribute to this activity in microsome from uninduced rats (Reik *et al.*, 1985). *In vivo* cimetidine inhibited pentoxyresorufin 0-dealkylase activity in microsomes from uninduced rats, but did not affect this activity in microsomes from phenobarbital-induced rats (Figure 5). Similarly, the anti-cytochrome P450IIC11 antibody inhibited pentoxyresorufin 0-dealkylase activity in

microsomes from uninduced rats, but had little or no effect on this activity in microsomes from phenobarbital-induced rats (Figure 20). Cytochromes P450IIB1/2 account for more than 90% of the pentoxyresorufin 0-dealkylase activity in hepatic microsomes from phenobarbital-induced rats (Lubet et al., 1985; Waxman et al., 1987; Dutton and Parkinson, 1989), but does not contribute to this activity in microsomes from uninduced rats (Waxman et al., 1987). In vivo cimetidine inhibited androstenedione formation in microsomes from uninduced rats, but did not affect this reaction in microsomes from phenobarbital-induced rats (Figure 17). The anti-cytochrome P450IIC11 antibody partially inhibited this reaction in microsomes from uninduced rats, but had little or no effect on this reaction in microsomes from phenobarbital-induced rats (Figure 24). Cytochromes P450IIB1/2 account for 60-70% of the formation of androstenedione in hepatic microsomes from phenobarbital-induced rats, but does not contribute to the formation of this metabolite in microsomes from uninduced rats (Reik et al., 1985). In vivo cimetidine inhibited aminopyrine N-demethylase activity to a greater extent in microsomes from uninduced than those from phenobarbital-induced rats (Figure 4). The anti-cytochrome P450IIC11 antibody partially inhibited this activity in microsomes from uninduced rats, but had little or no effect on this activity in microsomes from phenobarbital-induced rats (Figure 19).

4.1.6 A Possible Effect of Substrate on the Inhibition of Cytochrome P-450-Mediated Enzyme Activities by Cimetidine

There has been no systematic investigation of the inhibitory effect of cimetidine on specific cytochrome P-450 enzymes. Only one previous study has examined the effect of a single dose of cimetidine on hepatic microsomal cytochrome P-450-mediated enzyme activities in rats (Drew *et al.*, 1981). In that study, adult male rats, four per group, were uninduced or pretreated with either phenobarbital or 3-methylcholanthrene and sacrificed 2 h following a single intraperitoneal injection of cimetidine (150 mg/kg). Several hepatic microsomal enzyme activities were determined, including benzo[a]pyrene hydroxylase and 7-ethoxycoumarin O-deethylase activities. According to these investigators, cimetidine inhibited benzo[a]pyrene hydroxylase activity by 89%, but had no effect on 7-ethoxycoumarin O-deethylase activity or several non-specific enzyme activities, in microsomes from each of the three pretreatment groups.

The results from the study of Drew *et al.* (1981) are difficult to interpret. It has been shown in immunoinhibition experiments that cytochrome P450IA1 accounts for more than 80% of the benzo[a]pyrene hydroxylase activity in microsomes from rats induced with 3-methylcholanthrene (Ryan *et al.*, 1982b). This would lead one to conclude that cimetidine inhibits the catalytic

function of cytochrome P450IA1. However, the inhibition of benzo[a]pyrene hydroxylase activity by cimetidine is inconsistent with the lack of effect of cimetidine on 7-ethoxycoumarin O-deethylase activity. In microsomes from the 3-methylcholanthrene-induced rats, cytochrome P450IA1 accounts for 60-70% of the 7-ethoxycoumarin O-deethylase activity (Park *et al.*, 1982; Hietanen *et al.*, 1987). Therefore, if cimetidine inhibits cytochrome P450IA1, inhibition of 7-ethoxycoumarin O-deethylase activity by cimetidine should have been observed in the study conducted by Drew et al. (1981). As well, if cytochrome P450IA1 is the enzyme responsible for ethoxyresorufin O-deethylase activity in microsomes from β-naphthoflavone-induced rats, then inhibition of ethoxyresorufin O-deethylase activity by *in vivo* cimetidine should have been observed in microsomes from β-naphthoflavone-induced rats (Figures 7 and 9). The apparent discrepancy in the effect of *in vivo* cimetidine on the microsomal metabolism of benzo[a]pyrene, ethoxyresorufin and 7-ethoxycoumarin may be substrate-related. It has been proposed that two substrate-binding sites exist on cytochrome P450IA1 (Phillipson *et al.*, 1982; Kao and Wilkinson, 1987) and that benzo[a]pyrene and ethoxyresorufin occupy different binding sites on the enzyme (Kao and Wilkinson, 1987). One could then postulate that cimetidine inhibits the site used by benzo[a]pyrene, but does not inhibit the one used by ethoxyresorufin or 7-ethoxycoumarin.

Competition for substrate-binding sites leads to alternate substrate inhibition. However, the inhibition of benzo[a]pyrene hydroxylase activity by *in vivo* cimetidine is unlikely to be competitive. Due to the short elimination half-life (30-45 min) of cimetidine in rats, the concentration of cimetidine in the liver at 2 h after a single dose of the drug, as well as the concentration of cimetidine in isolated microsomes, is likely to be very much lower than the concentration of the substrate (83 μM) used in the assay. Furthermore, as shown in Figure 31, cimetidine, when administered *in vitro*, did inhibit ethoxyresorufin 0-deethylase activity in microsomes from β-naphthoflavone-induced rats. Future studies are required to re-examine the effect of *in vivo* cimetidine on cytochrome P450IA1-mediated substrate oxidation. The inconsistency in the effect of *in vivo* cimetidine on the microsomal metabolism of benzo[a]pyrene, 7-ethoxycoumarin and ethoxyresorufin indicates that the inhibitory effect of cimetidine may be both enzyme- and substrate-related.

4.2 INHIBITION OF CYTOCHROME P-450-MEDIATED ENZYME ACTIVITIES BY *IN VITRO* CIMETIDINE

The results from the studies in the present investigation with *in vivo* cimetidine indicate that differential inhibition of hepatic microsomal cytochrome P-450-mediated enzyme activities occurs following a single intraperitoneal injection of the drug to adult male rats.

It has been shown by other investigators that cimetidine, when added to rat hepatic microsomes *in vitro*, inhibits numerous enzymes activities (Table 4), many of which are not enzyme-specific. Since the concentration of cimetidine required for the *in vitro* inhibition of a microsomal cytochrome P-450-mediated enzyme activity is typically 100-1000 times higher than the serum concentration associated with inhibition of hepatic drug metabolism *in vivo* (Somoygi and Muirhead, 1987), it is apparent that the inhibition of cytochrome P-450 by *in vitro* cimetidine is not necessarily equivalent to that found following the *in vivo* administration of the drug to intact animals. Experiments were performed to determine whether *in vitro* cimetidine would differentially inhibit hepatic microsomal cytochrome P-450-mediated enzyme activities, in a manner similar to that observed following the *in vivo* administration of the drug to rats. Enzyme kinetic experiments were also performed to compare the type of inhibition of the cytochrome P450IIC11-mediated testosterone 2α-hydroxylase activity by *in vitro* and *in vivo* cimetidine.

Except for testosterone 7α-hydroxylase activity, *in vitro* cimetidine inhibited all the enzyme activities examined and the IC_{50} values were in the low millimolar range (Figures 29-38, Tables 18 and 19). The results obtained with the enzyme-specific activities indicate that *in vitro* cimetidine inhibits the catalytic function of

cytochromes P450IIB1/2, cytochrome P450IIC11 and cytochromes P450IIIA1/2, but not cytochrome P450IIA1. Thus, the pattern of the differential effect of *in vivo* cimetidine on cytochrome P-450-mediated enzyme activities was not observed with the *in vitro* administration of cimetidine.

In vitro cimetidine inhibited the cytochrome P450IIC11-mediated testosterone 2α-hydroxylase activity in microsomes from uninduced and phenobarbital-induced rats (Figure 32), consistent with the inhibition of this activity by *in vivo* cimetidine (Figures 10 and 18). Thus, cimetidine inhibits cytochrome P450IIC11 whether administered *in vitro* or *in vivo*. However, the observed inhibition of cytochrome P450IIC11 by *in vitro* and *in vivo* cimetidine may not result from the same inhibitory mechanism. The reason for this is that the IC_{50} values of the inhibition of the cytochrome P450IIC11-mediated testosterone 2α-hydroxylase activity by *in vitro* cimetidine in microsomes from uninduced and phenobarbital-induced rats were in the low millimolar range (Table 19), whereas the serum cimetidine concentration in the rat at 90 min after a single intraperitoneal dose of 150 mg/kg of cimetidine is in the low micromolar range (Reichen *et al.*, 1986). To further explore the inhibitory effects of *in vitro* and *in vivo* cimetidine on cytochrome P450IIC11, enzyme kinetic experiments were performed with testosterone 2α-hydroxylase. Based on Lineweaver-Burk plots of the data, the inhibition of testosterone 2α-hydroxylase

was competitive by *in vitro* cimetidine (Figure 39), but non-competitive by *in vivo* cimetidine (Figure 40), indicating that different mechanisms of inhibition are involved. Since it has been shown that cimetidine is metabolized to a small extent by hepatic microsomes (Zbaida *et al.*, 1984), there could be competition between cimetidine and testosterone for the substrate-binding site(s) on cytochrome P450IIC11. It is therefore possible that the observed competitive inhibition of testosterone 2α-hydroxylase by *in vitro* cimetidine reflects alternate substrate inhibition. However, with *in vivo* cimetidine, competitive inhibition of testosterone 2α-hydroxylase was not observed. This is not surprising for two reasons. First, the concentration of cimetidine in the hepatocytes at 90 min after a single dose of the drug is likely to be very much lower than the *in vitro* concentration required for inhibition to occur. Second, the concentration of the freely diffusible cimetidine is further diluted during the preparation of the microsomes. The fact that the inhibition of testosterone 2α-hydroxylase by *in vivo* cimetidine was non-competitive provides further evidence that different mechanisms are involved in the inhibition of cytochrome P-450 by *in vitro* and *in vivo* cimetidine.

In vitro cimetidine, at concentrations of up to 10 mM, did not affect the cytochrome P450IIA1-mediated testosterone 7α-hydroxylase activity in microsomes from uninduced and

phenobarbital-induced adult male rats (Figure 35). The lack of inhibition may be the result of a very low affinity of cimetidine for cytochrome P450IIA1. If so, at sufficiently high concentrations, cimetidine should inhibit this enzyme. Alternatively, cimetidine may not be able to bind to the substrate-binding site(s) on cytochrome P450IIA1. This would then suggest that the substrate-binding site(s) on cytochrome P450IIA1 are somehow different from those on the enzymes that are inhibited by *in vitro* cimetidine.

In summary, the specific pattern of the differential effect of *in vivo* cimetidine on cytochrome P-450-mediated enzyme activities does not occur when cimetidine is administered *in vitro*.

4.3 EFFECT OF PREINCUBATION ON THE INHIBITION OF CYTOCHROME P-450-MEDIATED ENZYME ACTIVITIES BY LOW CONCENTRATIONS OF CIMETIDINE

If cimetidine inhibits cytochrome P-450-mediated hepatic drug metabolism *in vivo* by a non-competitive mechanism, as suggested by the result shown in Figure 40, then this would explain why relatively low concentrations of cimetidine in man and in rats can inhibit drug clearance. However, if cimetidine inhibits cytochrome P-450 only by a non-competitive mechanism, then it should be a more potent inhibitor of cytochrome P-450-mediated enzyme activities *in vitro*. In fact, the inhibition of testosterone 2α-hydroxylase and several other cytochrome P-450-mediated

enzyme activities by *in vitro* cimetidine is competitive (Figure 39 and Table 4). Thus, it seems that more than one mechanism is involved in the inhibition of cytochrome P-450 enzymes by cimetidine. It is well-established that an inhibitor of cytochrome P-450 can act by more than one mechanism. For example, SKF 525-A inhibits cytochrome P-450 by alternate substrate inhibition and metabolite-intermediate complexation (Schenkman *et al.*, 1972; Buening and Franklin, 1976) and chloramphenicol inhibits cytochrome P-450 by alternate substrate inhibition and covalent binding to the apoprotein of cytochrome P-450 (Grogan *et al.*, 1972; Reilly and Ivey, 1979; Halpert, 1981). The involvement of different mechanisms in the inhibition of cytochrome P-450 by *in vivo* and *in vitro* cimetidine may explain the discrepancy in the selectivity of the inhibition by *in vivo* and *in vitro* cimetidine. To gain further insight into the inhibition of cytochrome P-450 by *in vitro* cimetidine, experiments were performed to determine whether the selective inhibition of microsomal testosterone oxidation by *in vivo* cimetidine can be observed following the preincubation of hepatic microsomes with low concentrations of cimetidine *in vitro* prior to the initiation of substrate oxidation.

In microsomes from uninduced rats, preincubation with 0.05 mM cimetidine and 1 mM NADPH for 15 min resulted in a decrease in testosterone 2α- and 16α-hydroxylase activities,

but did not affect testosterone 2β-, 6β- or 7α-hydroxylase activity or androstenedione formation, compared to the microsomes preincubated with NADPH for 15 min in the absence of cimetidine (Figures 46-52 and 53C). This pattern of inhibition was similar to that observed with *in vivo* cimetidine (Figure 53A), except for the lack of inhibition of androstenedione formation. With the preincubation of microsomes with cimetidine and NADPH, androstenedione formation was decreased by 17% compared to the microsomes preincubated with NADPH only (Figures 52 and 53C). However, this decrease was not statistically significant (p = 0.079). A statistically significant decrease might well be observed with a larger sample size since cytochrome P450IIC11 accounted for approximately 60% of the androstenedione formation in microsomes from uninduced rats (Figure 24). In the absence of preincubation, *in vitro* cimetidine, at this low concentration (0.05 mM), did not inhibit any of the testosterone oxidase activities (Figures 46-52). In microsomes from phenobarbital-induced rats, preincubation with 0.05 mM cimetidine and 1 mM NADPH for 15 min resulted in a decrease in testosterone 2α-hydroxylase activity, but did not affect testosterone 2β-, 6β-, 7α-, 16α- or 16β-hydroxylase activity or androstenedione formation, compared to the microsomes preincubated with NADPH in the absence of cimetidine for the same length of time (Figures 46-52 and 54C). Once again, this pattern of inhibition was

similar to that observed with *in vivo* cimetidine (Figure 54A). In the absence of preincubation, *in vitro* cimetidine at this low concentration (0.05 mM), did not inhibit any of the activities (Figures 46-52). Thus, with preincubation, *in vitro* cimetidine (0.05 mM) inhibits cytochrome P450IIC11, but not cytochrome P450IIA1, cytochromes P450IIB1/2 or cytochromes P450IIIA1/2. These results are consistent with the pattern of inhibition obtained with *in vivo* cimetidine.

Several important points are evident from the preincubation studies with *in vitro* cimetidine. First, the differential inhibition of microsomal testosterone oxidation by cimetidine was observed only with relatively low concentrations (0.05 mM or less) of cimetidine (Figure 41). At higher concentrations, the differential effect was not apparent. This illustrates the importance of performing the initial experiment to determine the concentration-response relationship (Figure 41). Second, the inhibition of testosterone 2α-hydroxylase activity by *in vitro* cimetidine (0.05 mM) required the presence of NADPH in the preincubation medium (Figure 43). When NADPH was absent from the preincubation medium but added just prior to the initiation of substrate oxidation, inhibition of testosterone 2α-hydroxylase activity at the low cimetidine concentration (0.05 mM) was not observed (Figure 43). The requirement for NADPH in the preincubation medium suggests that the observed inhibition is the result of a catalysis-

dependent process and not simply due to a time-dependent binding of cimetidine to an enzyme. Third, the preincubation of microsomes with only NADPH (in the absence of cimetidine) for 15 min followed by the usual 5 min of substrate oxidation did not result in a substantial reduction in any of the enzyme activities (Figure 42). For this reason, additional NADPH was not added just prior to the initiation of substrate oxidation. It could be argued that the observed decrease in the cytochrome P450IIC11-mediated testosterone 2α-hydroxylase activity in microsomes from uninduced and phenobarbital-induced rats and testosterone 16α-hydroxylase activity in microsomes from uninduced rats (Figures 46 and 50) was due to competition for the available NADPH among the enzymes that metabolize testosterone and possibly those that metabolize cimetidine. Cimetidine is metabolized, although to a small extent, by rat hepatic microsomes (Zbaida et al., 1984). However, a saturating concentration of NADPH was used in the assay. Furthermore, if NADPH does become rate-limiting, then one would likely observe a decrease in all the activities and this did not occur in the present experiments.

The effect of preincubation of hepatic microsomes with cimetidine and NADPH on cytochrome P-450 enzyme activities was investigated in three previous studies (Jensen and Gugler, 1985; Ioannoni et al., 1986; Rekka et al., 1988). Jensen and Gugler (1985) determined the effect of

preincubation on the inhibition of 7-ethoxycoumarin 0-deethylase activity by cimetidine in microsomes from uninduced adult male rats. In a time-course experiment, these investigators demonstrated an increase in the inhibition of this enzyme activity in microsomes preincubated for 10 min with cimetidine (0.25 mM) and a NADPH-generating system. A 20 min preincubation period did not result in any further increase in the extent of inhibition. The authors hypothesized that either cytochrome P-450 or cimetidine is activated during the preincubation of microsomes, resulting in ligand binding between cimetidine and cytochrome P-450 and the observed inhibition of the enzyme activity. However, the result from this experiment is difficult to interpret due to the lack of an appropriate control group. Since the samples from the "control" group in the study by Jensen and Gugler (1985) were not preincubated, the observed decrease in ethoxycoumarin 0-deethylase activity after preincubation may have been due to a breakdown of heme from the holoenzyme as a result of lipid peroxidation in the presence of NADPH (Levin *et al.*, 1973). In the present investigation, the decrease in the enzyme activities after preincubation was not due to an effect of NADPH on the holoenzyme since in the control group, the microsomes were preincubated with NADPH (Figures 46 and 50). Ioannoni *et al.* (1986) examined the effect of preincubation on the inhibition of morphine N-demethylase

activity by cimetidine with the purpose of determining whether cimetidine, or a metabolite, causes irreversible modification of substrate-binding sites on cytochrome P-450. Microsomes from adult male rats were preincubated with or without 0.5 mM cimetidine in the presence and absence of a NADPH-generating system for up to 15 min prior to the initiation of substrate oxidation. Morphine N-demethylase activity was decreased when the microsomes were preincubated with both cimetidine and the NADPH-generating system. However, a similar decrease in this activity occurred when the microsomes were preincubated with the NADPH-generating system in the absence of cimetidine. Therefore, under their conditions, preincubation did not enhance the inhibition of morphine N-demethylase activity by cimetidine. While Rekka et al. (1988) claimed that preincubation had no effect on the inhibition of the microsomal oxidation of ethoxyresorufin or tofenacine by cimetidine, their conclusion is questionable since the experimental protocol was not explained in sufficient detail and the data were not shown. As shown in Figures 53C and 54C, preincubation affected the enzyme activities specific for cytochrome P450IIC11, but not those specific for cytochrome P450IIA1, cytochromes P450IIB1/2 and cytochromes P450IIIA1/2. It is therefore possible that only certain cytochrome P-450 enzymes form a complex with a metabolite of cimetidine.

Jensen and Gugler (1985) reported that in rats treated with multiple doses of cimetidine (75 mg/kg intraperitoneally, four times daily for four days), there was a decrease in both the total cytochrome P-450 content and 7-ethoxycoumarin 0-deethylase activity compared to saline-treated rats. However, these decreases were not apparent after washing the microsomes from the cimetidine-treated rats with potassium ferricyanide. It has been shown with nitrogen-containing metabolite-intermediate forming agents such as SKF 525-A and triacetyloleandomycin that the oxidation of the ferrous heme iron by potassium ferricyanide dissociates the metabolite-intermediate complex, rendering the enzyme active again (Franklin, 1977; Mansuy, 1987). However, Jensen and Gugler (1985) did not monitor the time-dependent formation of a spectral peak in microsomes incubated with cimetidine and NADPH, which would be indicative of the formation of a metabolite-intermediate complex. Although cimetidine is classified as a compound that inhibits cytochrome P-450 by metabolite-intermediate complexation in two books (Gibson and Skett, 1986; Alvares and Pratt, 1990), there is as yet no definitive evidence that cimetidine, either *in vivo* or *in vitro*, forms a metabolite-intermediate complex with cytochrome P-450 enzymes. Further studies are needed to elucidate the mechanisms of inhibition of hepatic cytochrome P-450 enzymes by *in vivo* and *in vitro* cimetidine.

IMPLICATIONS

A novel finding from the present investigation is that, *in vivo*, cimetidine is an efficacious inhibitor of rat hepatic cytochrome P450IIC11, but that it does not inhibit several inducible cytochrome P-450 enzymes. When microsomes are preincubated with cimetidine and NADPH, the pattern of inhibition is the same as that observed following the *in vivo* administration of cimetidine. Investigators will be able to use this drug as an experimental agent in the field of cytochrome P-450 research. For example, to facilitate the understanding of the mechanism of the bioactivation of a drug, it may be necessary to inhibit cytochrome P450IIC11. Cimetidine can be used for this purpose. As well, analogs of cimetidine could be designed and synthesized with the ultimate goal of creating a specific inhibitor; that is, a compound which inhibits only one cytochrome P-450 enzyme. Such an agent would be useful in identifying the particular enzyme involved in the oxidation of a given drug or substrate. It would also be particularly valuable in studies in which the aim is to examine the function of a particular cytochrome P-450 enzyme in the metabolism of a given drug in an intact animal.

The mechanisms of inhibition of cytochrome P-450 by cimetidine are not fully understood. Up to now, the inhibitory action of cimetidine has been studied with

isolated microsomes and with substrates that are not enzyme-specific. In most of the *in vitro* microsomal studies with cimetidine, the compound has been added immediately prior to the initiation of substrate oxidation. In the absence of a preincubation step, any inhibition that is due to a catalysis-dependent process may not be able to occur and most of the observed inhibition may be due to another mechanism that is less important *in vivo*. With the finding that a particular cytochrome P-450 enzyme (i.e. cytochrome P450IIC11) is inhibited by cimetidine, investigators may now be able to use an enzyme-specific substrate and the purified enzyme to elucidate the mechanisms of inhibition by cimetidine.

Since cimetidine differentially inhibits rat hepatic cytochrome P-450 enzymes, it is also possible that human hepatic cytochrome P-450 enzymes have different susceptibilities to inhibition by this drug. Once the human hepatic cytochrome P-450 enzymes inhibited by cimetidine are documented, this compound may also be used as a probe for these enzymes in clinical drug metabolism studies. In addition, if preincubation of microsomes with cimetidine and NADPH *in vitro* can be shown to model the inhibition observed *in vivo*, then similar studies with human hepatic microsomes may make metabolic drug-drug interactions more predictable.

FUTURE STUDIES

6.1 STUDIES WITH RAT HEPATIC MICROSOMES

There is no definitive evidence that cimetidine, either *in vivo* or *in vitro*, forms a metabolite-intermediate complex with cytochrome P-450 enzymes. To determine whether cimetidine inhibits rat hepatic cytochrome P-450 by metabolite-intermediate complexation, the initial experiment would be to incubate microsomes (from uninduced adult male rats) with cimetidine and NADPH and to determine whether there is time-dependent formation of a spectral peak at approximately 448-456 nm, as occurs with other compounds that form metabolite-intermediate complexes (Pershing and Franklin, 1982). If the result from this experiment is positive, then one should proceed to determine whether dissociation or displacement of the metabolite-intermediate-cytochrome P-450 complex with a compound such as potassium ferricyanide or a displacer would render the enzyme catalytically active again (see Introduction, Section 1.1.5.2). For this purpose, testosterone 2α-hydroxylase activity can be used since the catalytic function of cytochrome P450IIC11 has been shown to be inhibited by cimetidine. In this experiment, cimetidine may be administered either *in vivo* or *in vitro*. If cimetidine is given as a single dose *in vivo*, the isolated microsomes

should be washed with potassium ferricyanide (or a displacer) prior to conducting the enzyme assay. If cimetidine is added *in vitro*, then its concentration and the time-course of the reaction must be optimized and a preincubation step incorporated to allow for the formation of the metabolite-intermediate-enzyme complex. Potassium ferricyanide (or a displacer) would then be added to the preincubation mixture for an appropriate period of time prior to the initiation of substrate oxidation. If the inhibition of testosterone 2α-hydroxylase activity by cimetidine is due to metabolite-intermediate complexation, then this activity should be higher in the potassium ferricyanide-treated group when compared to the appropriate control group. A working hypothesis would be that rat hepatic cytochrome P450IIC11, but not cytochrome P450IIA1, cytochromes P450IIB1/2 or cytochromes P450IIIA1/2, forms a complex with a metabolite of cimetidine.

If the results from the above experiments are positive, then a follow-up study would be to determine the chemical basis for the formation of the metabolite-intermediate-enzyme complex. Cimetidine has an amine group in its side chain. Other compounds with an amine group such as amphetamine and SKF 525-A have been shown to inhibit cytochrome P-450 by metabolite-intermediate complexation (Franklin, 1977). In these cases, the metabolite that complexes the enzyme is the nitrosoalkane derivative, which

is formed by demethylation of the amine group (Mansuy, 1987). Therefore, an initial experiment would be to determine whether the N-desmethylcimetidine metabolite has an inhibitory effect on cytochrome P-450; more specifically, the cytochrome P450IIC11-mediated testosterone 2α-hydroxylase activity.

Induction, suppression and inhibition of cytochrome P-450 enzymes can all occur following the chronic administration of a xenobiotic to rats. Cytochromes P450IA1/2 and cytochromes P450IIB1/2 are modestly induced in rats injected with multiple doses of cimetidine over several days (Ioannides et al., 1989). It is still not known whether suppression of cytochrome P-450 contributes to the decrease in an enzyme activity following chronic cimetidine administration to rats or to man. The effect of the chronic administration of cimetidine to rats on cytochrome P-450 can be explored further. For example, in rats treated with cimetidine for several days and sacrificed 24 h after the last dose, is there a decrease in an enzyme activity such as testosterone 2α-hydroxylase activity? If so, is it due to suppression and/or inhibition of cytochrome P450IIC11? To determine whether a suppressive effect exists, the level of hepatic cytochrome P450IIC11 can be determined by immunoquantitation and compared to that from saline-treated control rats. To determine whether there is inhibition of cytochrome P-450 as a result of metabolite-intermediate

complexation, the microsomes from the cimetidine-treated rats should be washed with potassium ferricyanide and testosterone 2α-hydroxylase activity determined. If the activity is higher in the potassium ferricyanide-washed microsomes compared to the appropriate control group, then this would be an indication that an inhibitory effect also exists.

An earlier study has indicated that cytochrome P450IA2 contributes to most of the ethoxyresorufin 0-deethylase activity in microsomes from uninduced rats (Kelley *et al.*, 1987). Recently, it has been shown that cytochrome P450IIC11 also contributes to this activity in microsomes from uninduced rats (Nakajima *et al.*, 1990). Therefore, it is uncertain whether the inhibition of ethoxyresorufin 0-deethylase activity by cimetidine is due to inhibition of the catalytic function of cytochrome P450IA2 or cytochrome P450IIC11 or both. The high-affinity phenacetin 0-deethylase activity in microsomes from uninduced, 3-methyl-cholanthrene-induced or isosafrole-induced rats is specific for cytochrome P450IA2 (Sesardic *et al.*, 1990b). Therefore, to examine the effect of cimetidine on cytochrome P450IA2, one can determine whether the drug inhibits phenacetin 0-deethylase activity in microsomes from uninduced and isosafrole-induced rats.

The demonstration that only certain hepatic cytochrome P-450 enzymes in rats are inhibited by cimetidine will allow

investigators in the field of cytochrome P-450 to use this drug to study the catalytic function of these enzymes. Analogs of cimetidine could be synthesized as a means to develop specific inhibitors of cytochrome P-450 enzymes. As well, in conjunction with site-directed mutagenesis, one could use cimetidine to study the regions of the rat hepatic cytochrome P450IIC11 that are critical for enzyme inhibition.

The potential inhibitory effect of a compound on cytochrome P-450 enzymes is often "screened" in *in vitro* microsomal experiments, using a substrate known to be catalyzed by these enzymes. The experimental conditions of the assays are such that any observable inhibition of the enzyme activity by the test compound is often associated only with a competitive mechanism of inhibition. As demonstrated in the present investigation, the observed inhibition of an enzyme activity by the addition of an inhibitor to microsomes *in vitro* does not necessarily reflect that which occurs following the *in vivo* administration of the compound to an intact animal. Therefore, for inhibitors which appear more potent *in vivo* than they do *in vitro*, it may be necessary to re-examine their inhibitory effects in an attempt to elucidate their mechanisms of inhibition *in vivo*.

Results from the present investigation indicate that cytochrome P450IIC11 is the major or sole catalyst involved

in the oxidative metabolic pathway of certain substrates; for example, pentoxyresorufin 0-dealkylation in microsomes from uninduced adult male rats. Cytochrome P450IIC11 is not expressed in the livers of female rats (Kamataki *et al.*, 1985; Waxman *et al.*, 1985). Yet, such substrates are oxidized by hepatic cytochrome P-450 enzymes in uninduced female rats. A question to be answered is which enzymes in livers of female rats are responsible for the oxidation of these substrates. Cytochrome P450IIC11 is developmentally regulated. Its level in livers of male rats is low prior to puberty, increases after puberty and becomes negligible in old age (Waxman, 1984; Kamataki *et al.*, 1985). In livers of 24 month-old male rats, the level of cytochrome P450IIC11 is negligible, whereas cytochrome P450IIC12, which is a "female-specific" enzyme, is expressed (Kamataki *et al.*, 1985). For substrates (drugs) that are oxidized by cytochrome P450IIC11, is this enzyme still involved in the oxidation of these substrates (drugs) in livers of old male rats? If not, does cytochrome P450IIC12 become the major catalyst in these cases? To answer these questions, immunoinhibition studies should be performed with the appropriate monospecific antibody (e.g. anti-cytochrome P450IIC11 and anti-cytochrome P450IIC12 antibodies) to determine the contribution of the enzymes to the oxidation of the substrate (drug).

6.2 STUDIES WITH HUMAN HEPATIC MICROSOMES

Based on the differential inhibition of rat hepatic cytochrome P-450 enzymes by cimetidine observed in the present investigation, it is possible that human hepatic cytochrome P-450 enzymes have different susceptibilities to the inhibitory effect of cimetidine. To investigate the effect of cimetidine on specific human hepatic cytochrome P-450 enzymes, an approach would be to preincubate human hepatic microsomes with low concentrations of cimetidine in the presence of NADPH prior to the initiation of substrate oxidation. Initially, an internal control experiment should be conducted with a substrate, the oxidative metabolism of which is known to be inhibited by cimetidine in human hepatic microsomes. The next step would be to select a drug, the clearance of which is impaired by cimetidine in man, and to determine whether preincubation of human hepatic microsomes with a low concentration of cimetidine and NADPH has an effect on the inhibition of the oxidation of this drug by cimetidine. In subsequent experiments, enzyme-specific activities will be used. These would include: a) phenacetin 0-deethylase activity for human cytochrome P450IA2 (Sesardic *et al.*, 1988); b) N-nitrosodimethylamine N-demethylase (Ishizaki *et al.*, 1991) or chlorzoxazone 6-hydroxylase activity (Guengerich *et al.*, 1991) for human cytochrome P450IIE1; c) testosterone 6β-hydroxylase activity for human cytochrome P450IIIA enzymes (Waxman *et al.*,

1988a); d) debrisoquine 4-hydroxylase activity for human cytochrome P450IID6 (Distlerath *et al.*, 1985).

In each case where one of the above enzyme activities is inhibited by cimetidine, the mechanism(s) of inhibition should be investigated. Experiments can be performed to determine whether a metabolite-intermediate is involved. This can be done by incubating human hepatic microsomes with cimetidine and NADPH and recording the time-dependent formation of a spectral peak at approximately 448-456 nm as well as performing the enzyme assays in the presence and absence of potassium ferricyanide. For these experiments, it is important to use liver tissue from surgical patients who are not taking drugs that are known to result in the formation of metabolite-intermediate complexes; for example, erythromycin, amiodarone and amphetamine.

An important study would be to evaluate whether the result obtained from a preincubation experiment with human hepatic microsomes and low concentrations of cimetidine can be used to model the effect of cimetidine on hepatic drug clearance in man. The approach to this series of experiments would be to use the drugs as the substrates in the *in vitro* enzyme assays. Human hepatic microsomes would be preincubated with low concentrations of cimetidine in the presence of NADPH prior to the initiation of drug oxidation. One group of drugs to be used would be those in which the clearance of the drug and the major metabolites have been

shown to be affected by cimetidine; for example, theophylline (Grygiel *et al.*, 1984; Cusack *et al.*, 1985; Vestal *et al.*, 1987). Another group of drugs to be used would be those in which these variables have been shown not to be affected by cimetidine; for example, tolbutamide (Dey *et al.*, 1983; Stockley *et al.*, 1986; Adebayo *et al.*, 1988).

SUMMARY AND CONCLUSIONS

7.1 STUDIES WITH CIMETIDINE

1. The *in vivo* administration of a single dose of cimetidine (150 mg/kg) to adult male rats, which were sacrificed 90 min after the injection, differentially affected the hepatic microsomal cytochrome P-450-mediated enzyme activities.

    A) *In vivo* cimetidine inhibited the enzyme activities specific for cytochrome P450IIC11. Evidence was also obtained suggesting that cimetidine inhibited cytochrome P450IA2; however, this requires further investigation.

    B) Indirect evidence also indicated that unidentified enzymes other than cytochrome P450IIC11 were inhibited by *in vivo* cimetidine in microsomes from uninduced adult male rats.

    C) In contrast, *in vivo* cimetidine treatment in adult male rats apparently did not affect the enzyme activities specific for cytochrome P450IIA1, cytochromes P450IIB1/2 or cytochromes P450IIIA1/2. It remains possible that the lack of inhibition of these activities is substrate-related.

    D) In some cases, the extent of inhibition by *in vivo* cimetidine depended on prior treatment with an

enzyme inducer. This can be explained by the increasing contribution to such activities by inducible enzymes which were not subject to inhibition by cimetidine.

2. The differential effect of *in vivo* cimetidine on cytochrome P-450-mediated enzyme activities was not observed when cimetidine was added to hepatic microsomes *in vitro* immediately prior to the initiation of substrate oxidation.

    A) *In vitro*, cimetidine, at concentrations of up to 10 mM, did not affect an enzyme activity specific for cytochrome P450IIA1. This is the first case of a cytochrome P-450 enzyme not inhibited by cimetidine either *in vivo* or *in vitro*. In contrast, *in vitro* cimetidine did inhibit the enzyme activities specific for cytochromes P450IIB1/2, cytochrome P450IIC11, cytochromes P450IIIA1/2 and possibly cytochrome P450IA2. The IC_{50} values for the various enzyme activities were in the range of 1.0 - 7.4 mM.

    B) In microsomes from uninduced rats, the cytochrome P450IIC11-mediated testosterone 2α-hydroxylase activity was inhibited competitively by *in vitro* cimetidine, but non-competitively by *in vivo* cimetidine.

3. Preincubation of hepatic microsomes with cimetidine and NADPH resulted in inhibition of cytochrome P-450 that was more characteristic of that observed with *in vivo* cimetidine.

    A) Preincubation of microsomes with 0.05 mM cimetidine in the presence of NADPH for 15 min resulted in the inhibition of the enzyme activities specific for cytochrome P450IIC11, but not those for cytochrome P450IIA1, cytochromes P450IIB1/2 or cytochromes P450IIIA1/2, similar to the pattern of inhibition observed with *in vivo* cimetidine. This differential effect with *in vitro* cimetidine (0.05 mM) did not occur in the absence of NADPH in the preincubation medium or without the preincubation step.

4. The results from the present investigation suggest that *in vivo*, cimetidine inhibits cytochrome P450IIC11 by a catalysis-dependent process in adult male rats. This is apparently by a mechanism different from the inhibition of cytochrome P-450 by cimetidine *in vitro* in the absence of a preincubation step. The precise mechanism of inhibition of cytochrome P-450 by cimetidine should be elucidated in future studies.

7.2 STUDIES WITH MONOSPECIFIC ANTI-CYTOCHROME P450IIC11 ANTIBODY

1. Testosterone 2α-hydroxylase activity was not only a good marker for cytochrome P450IIC11 in hepatic microsomes from uninduced adult male rats, but was also found to be a good marker for this activity in those microsomes from adult male rats treated with phenobarbital or dexamethasone.

2. Testosterone 16α-hydroxylase activity was a good marker for cytochrome P450IIC11 in hepatic microsomes from uninduced adult male rats. However, in those microsomes from adult male rats treated with phenobarbital or dexamethasone, cytochrome P450IIC11 was only a minor contributor to testosterone 16α-hydroxylase activity.

3. Cytochrome P450IIC11 accounted partially for the oxidation of testosterone to androstenedione in hepatic microsomes from uninduced and dexamethasone-induced, but not phenobarbital-induced, adult male rats.

4. Cytochrome P450IIC11 accounted for almost all of the pentoxyresorufin 0-dealkylase activity in hepatic microsomes from uninduced adult male rats. However, this enzyme did not appear to contribute to this activity in microsomes from adult male rats treated with phenobarbital.

5. Cytochrome P450IIC11 accounted partially for the aminopyrine N-demethylase activity in hepatic microsomes from uninduced adult male rats, but it did not appear to contribute to this activity in microsomes from rats treated with phenobarbital.

6. Cytochrome P450IIC11 did not appear to play a major role in the N-demethylation of erythromycin in hepatic microsomes from uninduced adult male rats.

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