EFFECT OF TAMOXIFEN ON HEPATIC CYTOCHROME P450 EXPRESSION

IN ADULT FEMALE RATS

by

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ABSTRACT

Tamoxifen, a widely used therapeutic agent in the treatment of breast cancer, is known to suppress growth hormone (GH) secretion in rats and consequently may alter hepatic levels of several cytochrome P450 (CYP) isozymes that are regulated by the pattern of GH secretion. The present study was undertaken to examine the effect of tamoxifen on the expression of hepatic CYP enzymes in the rat.

Tamoxifen was administered to nine adult male and female Long Evans rats at a dose of 5 mg per rat s.c. once daily for two consecutive days while control rats received the vehicle (peanut oil) only. Rats were killed five weeks after treatment and liver microsomes prepared. Administration of tamoxifen resulted in a significant decrease in mean weight gain in male and female rats compared to controls, but liver weight as a percent of body weight was not affected. Serum estradiol levels and total CYP content of hepatic microsomes were also unchanged. The profile of testosterone metabolites was not affected in tamoxifen-treated male rats but was significantly (p<0.05) altered in tamoxifen-treated female rats, indicating that tamoxifen's effects were sex-specific and hormone-mediated. Tamoxifen treatment significantly increased testosterone 6\beta-hydroxylase activity and significantly decreased testosterone 7 α -hydroxylase activity but did not significantly affect *p*-nitrophenol hydroxylase or pentoxyresorufin O-depentylase activities. Immunoquantitation studies revealed that tamoxifen treatment significantly decreased CYP2A1 levels but had no significant effect on CYP2C7, CYP2E1 or CYP3A levels in adult female rats. Thus, tamoxifen treatment caused a significant decrease in the expression of CYP2A1 and its associated monooxygenase activity, but had no apparent effect on the expression of CYP3A even though testosterone 6\beta-hydroxylase activity was significantly increased. Monoclonal antibodies against CYP3A1 and CYP3A2 were used to determine if either isozyme was present but neither protein was detected.

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In comparison, neonatal ovariectomy did not significantly affect hepatic microsomal CYP2A1, CYP2E1 or CYP3A protein levels but significantly decreased CYP2C7 protein levels, whereas adult estradiol treatment significantly increased hepatic expression of CYP2A1, CYP2C7 and CYP3A and significantly decreased expression of CYP2E1 in adult female rats. The data indicate that estradiol levels influence the expression of these isozymes.

In conclusion, the results demonstrate that brief treatment with tamoxifen has a prolonged effect on hepatic expression of CYP in the adult female rat and that this effect differs from that produced by neonatal ovariectomy. The mechanism by which tamoxifen alters CYP-mediated enzyme activity and protein levels is not apparent and requires further study.

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LIST OF ABBREVIATIONS

ANOVA	one-way analysis of variance
BCIP	5-bromo-4-chloro-3-indolylphosphate
BIS	N,N'-methylene-bis-acrylamide
BSA	bovine serum albumin
СҮР	cytochrome P450 protein or mRNA
DMBA	7,12-dimethylbenz(a)anthracene
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
GH	growth hormone
GHRH	GH-releasing hormone
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
IgG	immunoglobulin
i.p.	intraperitoneally
MSG	monosodium glutamate
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	<i>p</i> -nitroblue tetrazolium chloride
PAGE	polyacrylamide gel electrophoresis
PB	phenobarbital
PBS	phosphate buffered saline
<i>p.o.</i>	orally

PROD	pentoxyresorufin O-depentylation
<i>S.C</i> .	subcutaneously
SDS	sodium dodecyl sulphate
SRIH	somatotropin release-inhibiting hormone
TEMED	N,N,N',N'-tetramethyethylenediamine

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1. INTRODUCTION

Cytochromes P450 were first identified in 1958 by Klingenberg (1958) and Garfinkle (1958) when they reported that hepatic microsomes contained a reduced pigment that absorbed light maximally at 450 nm after binding to carbon monoxide. This pigment was subsequently characterized by Omura and Sato (1961) as a b-type cytochrome. Functionally, cytochromes P450 catalyze oxidation reactions by inserting a single atom of oxygen derived from O_2 into the substrate, which can be either a xenobiotic or an endogenous compound, making the substrate more hydrophilic and allowing for easier elimination from the body. Now, more than thirty years after their initial discovery, cytochromes P450 are known to constitute a superfamily of enzymes that are responsible for the first step in the biotransformation of numerous drugs, carcinogenic chemicals, pesticides and environmental pollutants, as well as steroids, fatty acids and prostaglandins. Consequently, the cytochromes P450 determine the duration of action of many drugs, as well as play a vital role in the metabolism of normal cellular components.

Each isozyme is present in the liver in different amounts and is uniquely affected by age, sex, diet and some disease states, as well as previous exposure to inducing chemicals. Changes in the levels of these enzymes are significant since they result in altered hepatic drug and steroid metabolism. The regulation of expression of some of these enzymes is through hormonal control. One aspect of this control system involves sex- and age-related differences in the metabolism of drugs and steroids. Cytochrome P450-mediated metabolism has been observed to vary among males and females in many species, with the greatest

differences reported in rats (Kato, 1974; Goble, 1975). The slower metabolic rate observed in female rats can be explained by the fact that they have 10 to 30% less total hepatic microsomal cytochrome P450 than their male counterparts. However, sex-related differences as large as 20-fold (Kamataki *et al.*, 1980) have been observed for rates of metabolism of some compounds, suggesting that there are sex-specific cytochrome P450 isozymes that contribute to microsomal metabolism as a whole (Waxman *et al.*, 1985). The expression of several hepatic forms of cytochrome P450 that are developmentally and/or sexually regulated appear to be affected either directly or indirectly by estrogens, androgens, and growth hormone (GH).

Our laboratory is interested in the mechanisms of hormonal regulation of hepatic cytochromes P450. Tamoxifen, which has antiestrogenic properties as well as effects on GH levels, is a useful compound to study the influence of hormones on hepatic cytochrome P450 expression. The present study investigates the effects of tamoxifen on various cytochrome P450 isozymes.

1.1. CYTOCHROME P450 SUPERFAMILY

The superfamily of cytochromes P450 is thought to have originated more than 3.5 billion years ago from an ancestral gene and is currently comprised of 221 identified genes (Nelson *et al.*, 1993). For classification purposes, the superfamily is divided into families and subfamilies based on amino acid sequence similarity. Isozymes grouped into a gene family have at least 40% amino acid sequence similarity and members of a subfamily are greater than 55% similar. Presently, the cytochrome P450 superfamily is comprised of 36 gene families, 12 of which exist in all mammals examined so far. These 12 mammalian

families are made up of 22 subfamilies and 17 of these subfamilies have been mapped in the human genome. The main mammalian hepatic drug-metabolizing enzymes are found in families 1 through 4.

1.1.1. Nomenclature

In the past, cytochromes P450 were named randomly by various investigators which made it hard to compare results reported from different laboratories. To alleviate this confusion a universal nomenclature system based on evolutionary relationships was established. Nelson *et al.* (1993) recommend that a cytochrome P450 gene or cDNA be denoted by the italicized root symbol "*CYP*" ("*Cyp*" for mouse), the family be indicated by an Arabic number, the subfamily be represented by a letter, and the individual gene be designated by an Arabic numeral. The same nomenclature is recommended for mRNA and protein in all species but a non-italicized format is used. All cytochromes P450 discussed will be referred to using the nomenclature of Nelson *et al.* (1993). As a reference, the rat cytochromes P450 investigated in this study are displayed in Table 1.1. with their corresponding trivial names.

Gene Symbol	Trivial Name
CYP1A Subfamily	
CYP1A1	c, βNF-B
CYP1A2	P-448, d, HCB
CYP2B Subfamily	
CYP2B1	b, PB-4, PB-B, PBRLM5
CYP2B2	3, PB-5, PB-D, PBRLM6
CYP2B3	IIB3
CYP2C Subfamily	
CYP2C6	PB1 k PB-C pTF2
011200	$RI M_{52} PB2 2C6$
CYP2C7	f RLM5h pTF1
CYP2C11	h M-1 16 α 2c UT-A
	RLM5 male $UT-2$
CYP2C12	i 158 2d UT-1 female
0112012	F-2
<i>CYP2C13</i>	+g, -g, RLM3, UT-5
CYP2E Subfamily	
CYP2E1	i. RLM6
	J,
CYP3A Subfamily	
CYP3A1	pcn, PCNa, 6β-4, pIGC2
CYP3A2	pcn2, PCNb/c, PB-1,
	6β-1/3

Table 1.1. Nomenclature of rat cytochromes P450 investigated in this study. (Adapted from Nelson *et al.*, 1993).

The major subfamilies of hepatic cytochromes P450 in mammals are briefly reviewed

below.

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1.1.2. Cytochrome P450 1A gene subfamily

The cytochrome P450 1 gene family contains genes 1A1 and 1A2 that are ubiquitous in mammals. There is speculation that these isozymes are essential for survival and perhaps play a key role in the metabolism of critical endogenous compounds (Gonzalez, 1989). Human CYP1A1 and CYP1A2 are 68% similar in their amino acid sequences (Ouattrochi et Rat P450c and mouse P1-450 have 78% and 80% sequence similarity, al., 1986). respectively, with human CYP1A1 (Sogawa et al., 1986; Jaiswal et al., 1985). Human CYP1A1 is thought to be mainly an extrahepatic cytochrome P450 and it is unclear as to whether it is inducible in the liver, while CYP1A2 is universally expressed in adult human liver (with a high degree of interindividual variation), but has not been detected in human extrahepatic tissues. In experimental animals CYP1A1 is expressed in both the liver and extrahepatic tissues. It is present constitutively at very low levels but is highly inducible by exposure to polycyclic aromatic hydrocarbons such as 3-methylcholanthrene. CYP1A1 metabolizes xenobiotics such as benzo(a)pyrene but has no known endogenous substrates (Kamataki et al., 1983; Sakaki et al., 1984). CYP1A2 displays high catalytic activity toward many arylamine compounds as well as the ability to metabolize potent promutagens derived from pyrolysates of amino acids and proteins (Kamataki et al., 1983).

1.1.3. Cytochrome P450 2A gene subfamily

The CYP2A subfamily has three members in the rat (hepatic CYP2A1, hepatic CYP2A2, and lung CYP2A3), two members in the mouse (Cyp2A4 and Cyp2A5), and one member in humans (CYP2A6). These isozymes demonstrate large differences in their substrate specificity and regulation. Human CYP2A6 is 85% similar to the rat lung CYP2A3

and less than 70% similar to the rat liver CYP2A1 and CYP2A2 forms (Yamano *et al.*, 1990).

1.1.4. Cytochrome P450 2B gene subfamily

The CYP2B subfamily consists mainly of phenobarbital (PB)-inducible isozymes. Rat CYP2B1 and CYP2B2 share 97% sequence similarity (Waxman and Walsh, 1982) but are only 77% similar to rat CYP2B3 (Labbe *et al.*, 1988) which is not inducible by phenobarbital. There is a PB-inducible form in rabbit and mouse, and human CYP2B6 shares 76% sequence similarity with rat CYP2B1 (Miles *et al.*, 1988).

1.1.5. Cytochrome P450 2C gene subfamily

In contrast to the CYP2B subfamily, the CYP2C subfamily consists of predominantly noninducible forms although a few are moderately PB-inducible (Gonzalez, 1989). Seven rat and eight rabbit forms have been identified and most of these isozymes are subject to developmental and sex-specific regulation (Nebert *et al.*, 1991). Several related human forms exist that are also constitutively expressed but are not sex-specific (Wrighton and Stevens, 1992).

1.1.6. Cytochrome P450 2D gene subfamily

The CYP2D subfamily has four rat forms that share 73 to 80% sequence similarity. CYP2D1 and CYP2D2 only have between 38% and 43% amino acid similarity with CYP2A1, CYP2B1, and CYP2E1 (Gonzalez, 1989). The important human form, CYP2D6,

is related to rat CYP2D1 (Nebert *et al.*, 1991). Humans who are poor metabolizers of debrisoquine are thought to lack CYP2D6 (Gonzalez *et al.*, 1988).

1.1.7. Cytochrome P450 2E gene subfamily

CYP2E1, a distinct, ethanol-inducible cytochrome P450, was first identified in rabbits (Koop *et al.*, 1982), followed by rats (Ryan *et al.*, 1985) and humans (Wrighton *et al.*, 1987). While only one gene, *CYP2E1*, exists in rats and humans, rabbits possess two genes (*CYP2E1* and *CYP2E2*) (Khani *et al.*, 1988). There is a good correlation between results of studies involving CYP2E1 in humans and rats which makes the rat a good model to study human CYP2E1 expression. CYP2E1 is an important isozyme because it is induced by many common small organic molecules such as ethanol, acetone and isoniazid, as well as by many physiological states including fasting, diabetes and obesity (Yang *et al.*, 1990).

1.1.8. Cytochrome P450 3A gene subfamily

This subfamily is responsible for the metabolism of various important clinical and toxicological agents including erythromycin, nifedipine, benzphetamine, lidocaine and aflatoxins (Watkins *et al.*, 1985; Guengerich *et al.*, 1986; Imaoka *et al.*, 1990; Ramsdell *et al.*, 1991). Its members are induced by steroids, macrolide antibiotics, imidazole antifungals, and phenobarbital (Gonzalez, 1988). The rat CYP3 family is thought to contain three or four proteins but currently only CYP3A1 and CYP3A2 have been identified. CYP3A1 is not expressed in untreated adult animals but it is induced by glucocorticoids, and CYP3A2 is only expressed in adult male rats but it is not induced by glucocorticoids (Gonzalez *et al.*,

1986). The human CYP3 family contains at least four genes (*CYP3A3*, *CYP3A4*, *CYP3A5*, and *CYP3A7*) (Nebert *et al.*, 1991).

1.1.9. Cytochrome P450 4A gene subfamily

Rat CYP4A1 was the first member purified (Tamburini *et al.*, 1984). This isozyme is induced by peroxisome proliferators such as clofibrate and demonstrates fatty acid hydroxylase activity. Other rat forms, as well as several rabbit forms, have also been identified (Gonzalez, 1989).

1.2. HORMONAL REGULATION OF CYTOCHROMES P450

In some animals, the extent and specificity of hepatic drug biotransformation is regulated qualitatively and quantitatively through hormonal control. The effects of different hormone levels are apparent when comparing the expression of individual cytochromes P450 in opposite sexes and various age ranges. There are definite sex-related differences in cytochrome P450 expression in many mammalian species, such as rats (Waxman *et al.*, 1985), mice (Noshiro and Negishi, 1988), hamsters (Sakuma *et al.*, 1993), and goats (van't Klooster *et al.*, 1993), but not humans (Wrighton and Stevens, 1992). These differences may be extreme, as demonstrated by CYP2C11 and CYP2C12 which are male-specific and female-specific isozymes, respectively, in the rat (Waxman *et al.*, 1985) or moderate, as shown by the female predominant CYP2A1 (Waxman *et al.*, 1990) which is present at twice the level in adult female rats as compared to adult male rats. In hamsters, one isozyme, CYP2C27, is male-specific in one organ (the kidneys) and male predominant in another (the liver) (Sakuma *et al.*, 1993). No human cytochrome P450 isozyme has been shown to be

expressed exclusively in one sex. The human CYP3A subfamily appears to be present at a higher level in females than males (Watkins *et al.*, 1989) but due to the large individual variation in cytochrome P450 content in the human population it is difficult to make any firm conclusions. Age-related differences in cytochrome P450 expression have been observed in many mammals, including man. For example, CYP2C7 is expressed at a very low level in three week old rats (about 0.5% of total cytochrome P450), but adult male and female rats express this isozyme as 7% and 14% of total cytochrome P450, respectively (Bandiera and Dworschak, 1992). In humans CYP3A7 comprises 30 to 50% of total fetal cytochrome P450 (Wrighton *et al.*, 1988; Wrighton and VandenBranden, 1989) but it has not been detected in the adult liver (Wrighton *et al.*, 1989; Wrighton *et al.*, 1990). Although most of the constitutively expressed cytochromes P450 are under some degree of hormonal control, each isozyme is uniquely regulated.

1.3. GROWTH HORMONE

1.3.1. Characterization of GH

It is thought that the differences in the pattern of GH secretion between immature and mature, as well as male and female rats are responsible for the variations in cytochrome P450 levels. Almost twenty years ago, Gustafsson *et al.* (1976) determined that the factor responsible for the sex-related difference in drug metabolism is contained in a secretion from the pituitary gland, but that the pituitary gland itself is not sex-differentiated. Mode *et al.* (1983) then established that this pituitary factor is GH. The delay in identifying GH as the sex-differentiating factor was due to the observation that the mean serum GH level in male and female rats is very similar. The plasma GH level in rats is high at birth (approximately

50 ng/mL), decreases for the next 15 days, and then increases again (Wehrenberg, 1986; Ojeda and Jameson, 1977; Rieutort, 1974; Blazquez et al., 1974; Walker et al., 1977). It was later realized that it is the adult pattern of GH secretion that is sex-differentiated and that this is what accounts for the changes in metabolism. Eden (1979) observed that before the age of 22 days the pattern of GH secretion is the same in male and female rats, but by 30 days of age the sex-differentiated patterns have been established. Figure 1.1. displays the secretory profiles of GH in female and male rats at 22, 30, 45, and 90 days of age (Eden, 1979). GH is present only intermittently in the plasma of adult male rats and its secretion is known as "pulsatile" with distinct peaks and troughs (periodicity of approximately 3.3 hours or 7 surges/day) (Tannenbaum, 1976), while adult female rats display a "continuous" higher frequency/lower amplitude pattern (1-2 pulses/2 hours) that maintains GH levels greater than or equal to 10 to 20 ng/mL of plasma (Waxman et al., 1991). Adult male rats have trough periods with GH levels below detection limits (less than 1 ng/mL) for 60 to 120 minutes as compared to adult female rats with GH trough levels greater than 5 ng/mL for 15 to 30 minutes (Eden, 1979; Tannenbaum, 1976). This sex-differentiated pattern of GH secretion is not unique to rats, but is characteristic of several animal species, including humans (Jansson et al., 1985b; Devesa et al., 1991).



Figure 1.1. Representative 6-h secretory profiles in female and male rats at 22, 30, 45, and 90 days of age. Sequential blood samples were obtained from implanted cannulae at 30-min intervals. *Arrows* indicate plasma GH levels > 300 ng/ml (305 and 314 ng/ml). (Taken from Eden, 1979.)

1.3.2. Regulation of GH secretion

The GH (or somatotrope) axis is affected by many factors as it consists of the central nervous system, hypothalamus, anterior pituitary, and peripheral tissues (Kerrigan and Rogol, 1992). The pattern of GH secretion described previously is mediated by episodic increases in the release of GH-releasing hormone (GHRH) and decreases in the release of somatotropin release-inhibiting hormone (SRIH). Kracier *et al.* (1988) observed that the amplitude of the

GH secretory episodes is determined by the amount of GHRH released and that the frequency and duration are determined by the pattern of SRIH secretion.

Clinically, it has been observed that human GH physiology is dramatically affected by the pubertal serum levels of the sex steroid hormones (either endogenous or exogenous) through augmentation of GH pulse amplitude. Hindmarsh *et al.* (1987) have reported that a strong association exists between GH pulse amplitude and childhood growth rate. Direct knowledge about human GH physiology has been limited due to many practical and ethical issues but basic science investigations are essential to understanding the mechanisms involved. Human GH secretion from the somatotrope is influenced by the hypothalamus in the same manner as in rats, making the rat an excellent model to study GH regulation (Kerrigan and Rogol, 1992).

Many experiments have been performed in an attempt to understand how the pattern of GH secretion is regulated. Jansson and Frohman (1987a and 1987b) neonatally gonadectomized male and female rats and observed that the GH secretory patterns displayed were affected. Figure 1.2. displays individual plasma GH profiles in neonatally gonadectomized adult male rats in comparison to those of neonatally sham-operated adult male rats and Figure 1.3. shows the effects of neonatal gonadectomy on characteristics of the GH secretory pattern in adult male rats (Jansson and Frohman, 1987a). Figure 1.4. displays the plasma GH profiles of neonatally ovariectomized adult female rats in comparison with those of adult male rats and adult female rats and Figure 1.5. shows the effects of neonatal ovariectomy on characteristics of the GH secretory pattern in adult female rats (Jansson and Frohman, 1987b). The males demonstrated a 50 to 75% decrease in GH pulse amplitude and an increase in GH trough concentrations (Jansson and Frohman, 1987a; Birge *et al.*,







Figure 1.3. Effects of neonatal gonadectomy (n=7) on characteristics of the GH secretory pattern in adult male rats. Controls were sham-operated (SHAM; n=12). *, P < 0.05 vs. SHAM. (Taken from Jansson and Frohman, 1987a.)



Figure 1.4. Plasma GH profiles in individual adult female rats after neonatal gonadectomy in comparison to those of adult male and female rats after neonatal sham gonadectomy. Plasma GH values greater than 500 ng/mL are shown in *parentheses*. GH pulses are indicated by *arrows*. The baseline GH levels are shown as *interrupted lines*. (Taken from Jansson and Frohman, 1987b.)



Figure 1.5. Effects of neonatal gonadectomy (n=9) on characteristics of the GH-secretory pattern in adult female rats. Male (n=6) and female (n=7) controls were sham operated. *, P < 0.05 vs. sham-operated males; †, P < 0.05 vs. sham operated females. (Taken from Jansson and Frohman, 1987b.)

1967) and the females displayed a decrease in GH trough values during adulthood (Jansson and Frohman, 1987b), as compared to their sham-operated counterparts. Even though neonatal ovariectomy suppressed the baseline plasma GH in adult female rats to a level that was not significantly different from that in male rats, the plasma GH profiles were still obviously different from those in normal male rats (Jansson and Frohman, 1987b). Administering estrogen to male rats has also been shown to lower pituitary GH content (Birge *et al.*, 1967). The effects of neonatal orchidectomy on male rats were shown to be reversible by testosterone replacement therapy (Jansson and Frohman, 1987a). If testosterone was only administered neonatally, the GH pulses were of shorter duration and increased frequency, but if testosterone was administered both neonatally and during adulthood, the GH secretory pattern displayed was indistinguishable from normal males, the same as males which received testosterone treatment during adult life only. This suggests that the presence of testosterone during adulthood plays both a corrective as well as a maintenance role (Wehrenberg and Giustina, 1992). Studies with female rats showed that their GH secretory pattern can become totally male-like by neonatal gonadectomy followed by testosterone replacement therapy (Jansson et al., 1985c), but if the ovaries are still intact the pattern of GH secretion in adult female rats is unaffected by the exogenous neonatal androgen treatment (Jansson and Frohman, 1987b). These studies suggest that the proper hormonal environment during the neonatal and adult period is important for the development of the characteristic secretory patterns of GH in both adult male and female rats.

1.4. EXPERIMENTAL MODELS USED TO UNDERSTAND CYTOCHROME P450 REGULATION

Studies have shown that cytochromes P450 are indirectly regulated by the gonadal hormones through their effects on the hypothalamo-pituitary axis and its control of GH secretion (Gustafsson *et al.*,1983; Waxman, 1988; Jansson and Frohman, 1987a). Animal models used to help understand the key components of the GH secretory pattern responsible for the regulation of the sex-specific cytochromes P450 are: neonatally castrated male rats, neonatally ovariectomized female rats, dwarf rats, monosodium glutamate (MSG)-treated rats, and hypophysectomized rats with GH replacement therapy.

1.4.1. The neonatally castrated male rat model

Hepatic steroid and drug metabolism in the rat has been known to be sex-dependent for many years. The most obvious hormones thought to be responsible for this phenomenon are the sex steroids. Neonatal orchidectomy has been shown to drastically reduce serum testosterone levels (Bandiera and Dworschak, 1992) as well as markedly suppress (50 to 75%) GH pulse height, pulse duration and mean plasma GH concentration, and significantly increase baseline GH levels (see Figure 1.3.) (Jansson and Frohman, 1987a). Kamataki *et al.* (1985) and Waxman *et al.* (1985) demonstrated that male-specific CYP2C11 is suppressed in neonatally castrated male rats while female-specific CYP2C12 is partially expressed. They also showed that this effect was reversed by neonatal testosterone administration. In a follow-up study, Shimada *et al.* (1987) observed that the expression of CYP2C11 is almost completely restored in neonatally castrated rats by treatment with testosterone during adulthood. The hepatic levels of another male-specific isozyme, CYP2A2, is dramatically

decreased when male rats are castrated neonatally, but is reduced by only 25% when rats are castrated at puberty (Waxman *et al.*, 1988), while expression of male-specific CYP2C13 is suppressed in neonatally castrated adult male rats but not in male rats that were castrated during puberty. CYP2C12, a female-specific form, is expressed in male rats castrated at birth at a level that is approximately 25 to 30% that found in normal female rats (Dannan *et al.*, 1986). All of these studies demonstrate that testosterone has an effect on the expression of some cytochrome P450 isozymes either directly, or indirectly through its effects on GH secretion.

1.4.2. The neonatally ovariectomized female rat model

Neonatal ovariectomy has been shown to lower serum estradiol levels (Jansson and Frohman, 1987b) and although it only minimally decreases mean GH levels it affects the pattern of GH secretion (see Figure 1.5.) (Jansson and Frohman, 1987b). The expression of CYP2A1 is masculinized in female rats ovariectomized at birth and this effect is reversible by estradiol replacement (Waxman *et al.*, 1989). Neonatally ovariectomized adult female rats expressed CYP2C7 at levels about half of those found in intact adult female rats but the level of this isozyme was restored by treatment with estradiol during adulthood or puberty (Bandiera and Dworschak, 1992). CYP2C12, a female-specific isozyme, is expressed at only 30% of its normal level in neonatally ovariectomized female rats (Dannan *et al.*, 1986). Hepatic levels of female predominant CYP2E1 are significantly increased in female and male rats that were neonatally gonadectomized (Waxman *et al.*, 1989). These studies indicate that the female-related cytochromes P450 are either affected directly by estrogen, or indirectly through its effects on the GH secretory pattern.

1.4.3. *The dwarf rat model*

A colony of dwarf (GH-deficient) rats homozygous for an autosomal recessive mutation that reduces pituitary GH levels to 5% of normal levels has been established by Charlton et al. (1988). The circulating GH levels in these rats are barely detectable (less than 5 ng/mL), yet dwarf rats do grow larger than hypophysectomized rats, although both dwarf and hypophysectomized rats are considerably smaller than normal intact rats (Charlton et al., 1988). Dwarfism is a result of a selective defect in GH synthesis and storage as indicated by the fact that other pituitary hormones are present at normal or slightly elevated levels (Charlton et al., 1988; Bartlett et al., 1990). Bullock et al. (1991) measured various protein levels and enzyme activities in hepatic microsomes from immature and mature male and female dwarf rats and found that the magnitudes of the sex-dependent and age-related differences in CYP2A1-, CYP2A2-, CYP2B1-, CYP2C11-, and CYP2C12-mediated enzyme activities and protein levels were comparable to those observed in normal rats, suggesting that an extremely low GH level does not affect the expression of these isozymes. Further investigations revealed that the sexually dimorphic pattern of GH secretion in dwarf rats is identical to that of normal rats except for a reduction in the pulse amplitude (Jeffrey et al. 1990; Legraverend et al., 1992). These results suggest that it is the GH secretory pattern that regulates the levels of these isozymes and not the amount of GH itself.

1.4.4. The MSG-treated rat model

As a nonsurgical means of reducing adult GH levels, rats can be injected neonatally with MSG. Within two to four days of neonatal administration of MSG, destruction of 80 to 90% of the neurons located in the arcuate nucleus of the hypothalamus occurs and causes

permanent and specific loss of GH releasing factor, but somatostatin secretion is not affected (Bloch et al., 1984; Olney, 1981; Lemkey-Johnston and Reynolds, 1974). This results in a dramatic (90%) inhibition of GH secretion in both sexes but a marked reduction of pulse frequency in females only (Maiter, 1991). This method is advantageous over hypophysectomy for studying the effects of suppressed GH levels on hepatic cytochrome P450 expression because the serum levels of other pituitary-dependent hormones, such as corticotropin (Krieger et al., 1979), prolactin (Terry et al., 1981; Millard et al., 1982), thyroid-stimulating hormone (Nemeroff et al., 1977), luteinizing hormone, folliclestimulating hormone, testosterone and estradiol (Bakke et al., 1978; Rose and Weick, 1987), are largely unchanged. Waxman et al. (1990) used MSG-treatment to selectively study the effect of dramatically reduced GH levels on specific cytochrome P450 isozymes. When male and female rats were treated with 2 mg MSG/g body weight the pulse amplitude and mean plasma GH levels were reduced by 90 to 95% but the pattern of GH secretion remained typically masculine and feminine, respectively. In the males, the hepatic level of malespecific microsomal CYP2C11 protein was increased slightly above that of normal intact males, while in the females the hepatic level of female-specific microsomal CYP2C12 level was unaffected. These experiments indicate that the expression of CYP2C11 and CYP2C12 are not regulated by the amplitude of the GH pulse or the mean plasma level of GH (Shapiro et al., 1989). Administration of 4 mg MSG/g body weight decreased serum GH to undetectable levels (less than 2 ng/mL). This treatment caused male rats to become feminized in that no CYP2C11 was expressed and CYP2C12 was expressed at about 20% of the intact female level. In female rats, CYP2C11 remained unexpressed and CYP2C12 was

maintained at a normal or slightly higher level (Waxman *et al.*, 1990). Expression of CYP3A2, another male-specific isozyme, was fully suppressed in adult male rats and remained undetectable in adult female rats neonatally treated with 4 mg MSG/g body weight. Expression of CYP2E1 was not affected in males and only slightly elevated in females treated with 2 mg MSG/g body weight, but it was increased 2-fold in male and female rats treated with 4 mg MSG/g body weight. Treatment with 2 mg MSG/g body weight had no effect on the level of expression of CYP2A1 in male or female rats, but at a dose of 4 mg MSG/g body weight, this isozyme was elevated about 2-fold in male rats and only slightly increased in female rats. These results show that even when GH levels are reduced by up to 95% (2 mg/g MSG-treatment) cytochromes P450 are minimally, if at all, affected, yet when GH levels are undetectable (4 mg/g MSG-treatment) hepatic expression of some isozymes is significantly altered.

1.4.5. The hypophysectomized rat model

Waxman *et al.* (1991) examined the importance of the GH secretory pattern by using an external pump to administer pulses of rat GH to hypophysectomized rats. The frequency and the amplitude of the pulses were varied to determine the effect on CYP2C11. They observed that rat GH delivered as 2, 4, or 6 pulses per day stimulated the hepatic expression of CYP2C11, while GH delivered as 7 pulses per day did not. Female-specific testosterone 5α -reductase activity was stimulated by 7 pulses/day, although to a much less degree than by continuous GH infusion. The dramatic change in the level of CYP2C11 as a result of only a 35 minute difference in interpulse interval suggests that a minimal plasma GH trough time is essential for masculinization of GH-regulated, male-specific proteins. The investigators concluded that the hepatic expression of CYP2C11 was stimulated by GH at both physiological and nonphysiological pulse amplitudes, durations and frequencies, as long as an interpulse interval of no detectable GH was maintained for at least 2.5 hours.

The results from these models have led scientists to hypothesize that "it is neither the amplitude nor the frequency of the GH pulses that is recognized as male or female by the hepatocyte, but rather the complete and prolonged suppression in males, or the persistence in females, of circulating GH during the trough period after a GH surge" (Legraverend *et al.*, 1992).

1.5. TAMOXIFEN

Tamoxifen, a nonsteroidal partial competitive antagonist of the estrogen receptor, was first synthesized in 1966 (Harper and Walpole, 1967) and has become the leading drug in the fight against hormone-dependent breast cancer. In the past, tamoxifen therapy has been shown to increase the disease-free survival of breast cancer patients (Costa and Jordan, 1991), and clinical trials are underway at present to determine its potential as a prophylactic agent in women with a high risk of developing this disease (Jordan, 1990; Fentiman, 1990). Since tamoxifen is only cytostatic, and not cytotoxic, it must be used for long periods of time.

Tamoxifen is a triphenylethylene derivative that exhibits both estrogen agonist and antagonist activity depending on the animal species. In humans and the rat, tamoxifen is a

partial estrogen/antiestrogen, while it is a full estrogen in the mouse and a pure antiestrogen in the chick (Harper and Walpole, 1967; Terenius, 1971; Sutherland *et al.*, 1977).

The main mechanism of action of tamoxifen is thought to be through preventing growth-stimulating estrogen from binding to the estrogen receptor, but 10 to 15% of estrogen receptor negative breast cancer patients benefit from this drug, indicating that tamoxifen has estrogen receptor-independent effects. Tamoxifen appears to have several mechanisms of action as suggested by its estrogen agonist and antagonist properties as well as estrogen receptor-independent activities. Studies have shown that tamoxifen decreases the risk of coronary heart disease and has estrogen-like effects on bone (Dewar et al., 1992; Wright et al., 1993). Tamoxifen also causes estrogen-like effects on plasma proteins in all women as demonstrated by the increase in sex hormone-, cortisol-, and thyroxine-binding globulins. The level of circulating estradiol is greatly increased in premenopausal women on tamoxifen therapy but remains low in tamoxifen-treated postmenopausal women (Love, 1989). Prolactin, which plays a key role in normal breast differentiation and in rodent breast cancer, has been shown to be decreased or unaffected by tamoxifen treatment (Osborne and Arteaga, 1990). Since prolactin secretion is normally potentiated by estradiol, tamoxifen is suspected of blocking estrogen receptors at the hypothalamic-pituitary level and interfering with prolactin release (Groom and Griffiths, 1976). This suspicion is supported by observations that most of the effects of prolactin are inhibited by nanomolar concentrations of triphenylethylenes. The mechanism of action of tamoxifen is still being investigated but three possibilities are: (i) blocking stimulation by estradiol, (ii) causing stimulation at sites where estradiol blocks, and (iii) estrogenic effects (van den Koedijk et al., 1994).
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Tamoxifen is metabolized extensively in the mouse, rat, rhesus monkey, and man to 4-hydroxytamoxifen, N-desmethyltamoxifen, metabolite D (3,4-dihydroxytamoxifen), metabolite E (loss of basic side chain), metabolite Y (deaminated derivative), metabolite Z (N-desdimethyltamoxifen), and tamoxifen N-oxide (see Figure 1.6.) (Jordan, 1984). Virtually no tamoxifen is eliminated as intact drug. The metabolites are excreted mainly as conjugates in the bile. Urinary excretion is greater in primates than in rodents but only plays a minor role in the elimination of tamoxifen. Tamoxifen has a half-life of ten days in rats and seven days in humans (Robinson and Jordan, 1988). Two metabolites of interest are 4hydroxytamoxifen and N-desmethyltamoxifen. 4-Hydroxytamoxifen is highly active and is present in humans at very low concentrations (at least 25 times lower than tamoxifen) while N-desmethyltamoxifen has low activity and is present at concentrations about 50% greater than tamoxifen. Like tamoxifen, 4-hydroxytamoxifen is a partial agonist in the rat and a full agonist in the mouse. 4-Hydroxytamoxifen binds to the estrogen receptor with an affinity at least as great as that of estradiol, which is 20 to 30 times higher than tamoxifen's affinity for the estrogen receptor. The dissociation rate of 4-hydroxytamoxifen from the receptor is very slow like estradiol and unlike tamoxifen. Investigators have reported 4-hydroxytamoxifen to be anywhere from eight times more potent than tamoxifen to being equipotent to tamoxifen in vivo. 4-Hydroxytamoxifen has a much shorter duration of action in vivo than tamoxifen. N-desmethyltamoxifen is a partial agonist in the rat. It has a low binding affinity for the estrogen receptor and it is less potent than tamoxifen. Ninety-nine percent of a dose of tamoxifen and its metabolites are found in peripheral compartments, at relative quantities of 1, 0.01, and 1.2 for tamoxifen, 4-hydroxytamoxifen, and N-desmethyltamoxifen, respectively.



Figure 1.6. Metabolites of tamoxifen in animals and humans. Adapted from Jordan (1984).

Jordan (1976) demonstrated that tamoxifen is able to inhibit the growth of 7,12dimethylbenz(a)anthracene (DMBA)-induced mammary tumours in the rat using a dosing regimen previously reported to have long term anti-estrogenic effects (5 mg tamoxifen subcutaneously (*s.c.*) once daily for two consecutive days) (Emmens, 1971). Active

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metabolites of tamoxifen have been reported to persist in tissues for more than eight weeks (Lien et al., 1991). Tamoxifen and 4-hydroxytamoxifen (its active metabolite) also displayed estrogenic activity by stimulating uterine growth in ovariectomized rats and mice (Jordan et al., 1978). Tannenbaum et al. (1992) recently demonstrated that tamoxifen, when administered in the same manner used by Jordan (1976) to convey antineoplastic activity. displays potent inhibitory effects on GH secretion. The six hour plasma GH profiles in tamoxifen-treated adult male and female rats are displayed in Figure 1.7. and the GH peak amplitude and mean six hour plasma GH level in tamoxifen-treated adult male and female rats are shown in Figure 1.8. (Tannenbaum et al., 1992). These investigators showed that both the amplitude of spontaneous secretory bursts and the mean six hour plasma levels of GH were dramatically reduced in both sexes as compared to their respective controls for up to seven weeks post treatment. The inhibitory effect of tamoxifen on GH secretion has been demonstrated to be caused, at least partially, by an increased release of endogenous somatostatin (Tannenbaum et al., 1992) but it is not known if this effect is related to tamoxifen's antineoplastic activity.

Administration of tamoxifen does not exactly mimic the effect of either hypophysectomy or gonadectomy in terms of its suppression of GH levels. Hypophysectomy completely eliminates GH secretion and gonadectomy only minimally decreases mean GH levels (Jansson and Frohman, 1987a; Jansson and Frohman, 1987b; Shulman *et al.*, 1987). The tamoxifen treatment used in the study by Tannenbaum *et al.* (1992) was reported to have effects on GH secretory dynamics that were common with those of gonadectomy. In adult male rats, both treatments significantly suppress GH peak amplitude and mean six hour plasma GH levels (Tannenbaum *et al.*, 1992; Painson *et al.*, 1992) as compared to those



Figure 1.7. Individual representative 6-h plasma GH profiles in a peanut oil-injected control male and female rat compared to those in tamoxifen-treated male and female rats, respectively, at varying times after injection. (Taken from Tannenbaum *et al.*, 1992.)



Figure 1.8. Cluster analysis of GH secretory dynamics in male and female rats administered tamoxifen. Each *bar* represents the mean + SE; the number of animals in each group is shown in *parentheses.* a, P < 0.01; b, P < 0.001; c, P < 0.02 (compared to their respective peanut oil-injected controls). (Taken from Tannenbaum *et al.*, 1992.)

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of normal adult male rats. In adult female rats, both treatments significantly decrease baseline GH levels and mean six hour plasma GH levels (Tannenbaum *et al.*, 1992; Jansson and Frohman, 1987b) as compared to those of normal adult female rats. The similarity observed between the effect of tamoxifen treatment and that of reducing circulating estradiol levels by removal of the ovaries suggests that tamoxifen may act by blocking estradiol receptors in the hypothalamic-pituitary axis (Tannenbaum *et al.*, 1992). However, the effect of tamoxifen treatment on GH secretion is more pronounced than that of gonadectomy. The mean serum levels of GH in male and female adult rats five weeks after dosing with tamoxifen were approximately 30% and 20%, respectively, of mean serum GH levels in control rats.

1.6. HYPOTHESIS

Our working hypothesis was that tamoxifen, through its antiestrogenic or GH lowering effects, would alter the levels of hepatic cytochromes P450 that are regulated by estradiol or GH. Agents that cause severe suppression of GH levels, or that reduce levels of the sex hormones or otherwise block their action, can produce prolonged changes in the expression of hepatic cytochrome P450 isozymes. Alternately, the prolonged inhibition of estrogen activity in tamoxifen-treated female rats may, by itself, be sufficient stimulus for altering hepatic metabolism. There is also the possibility that tamoxifen might alter cytochrome P450-mediated activity without altering protein levels, via a mechanism such as competitive or non-competitive inhibition. To investigate these possibilities the expression of hormonally regulated cytochromes P450, specifically rat hepatic CYP2A1, CYP2B1/CYP2B2, CYP2C7, CYP2C11, CYP2E1 and CYP3A, were examined.

1.7. SEXUALLY DIFFERENTIATED CYTOCHROMES P450 INVESTIGATED IN THIS STUDY

The current state of knowledge regarding the regulation of the isozymes investigated in this study is briefly reviewed below.

1.7.1. Cytochrome P450 2A1

CYP2A1 is a female predominant isozyme (Waxman et al., 1990) that demonstrates high testosterone 7 α -hydroxylase activity (Wood *et al.*, 1983; Thomas *et al.*, 1981; Sonderfan et al., 1987). It is present in neonatal male and female rats and its level peaks at approximately two weeks of age. From about two to eight weeks of age the microsomal concentration of this isozyme decreases in males to about one fourth of the adult female level (Waxman et al., 1985; Imaoka et al., 1991). It comprises 3% and 6% of total cytochrome P450 in untreated adult male and female rats respectively, and is moderately inducible (elevated approximately 5-fold) by treatment with 3-methylcholanthrene (Thomas et al., 1981). Waxman et al. (1989) investigated the role that the gonadal hormones play in the expression of CYP2A1. They observed that when male rats are castrated at birth or during adulthood their adult level of CYP2A1 is partially feminized (increased approximately 2.5fold) relative to that of controls. This effect is substantially reversed by testosterone, but only when administered during adulthood. In female rats ovariectomized at birth, the level of CYP2A1 during adulthood was similar to that observed in male rats that were castrated at birth, and this was shown to be reversible by estradiol replacement. Thus, the sex hormones, estrogen and testosterone, were determined to have a positive and a negative influence, respectively, on the expression of CYP2A1 (Waxman *et al.*, 1989). Since the effects of gonadal hormones on hepatic enzyme levels are often mediated by the pituitary, hypophysectomy studies were performed. Hypophysectomized adult male rats were shown to express CYP2A1 at the same level as prepubertal males, but levels in hypophysectomized adult females were not affected (Waxman *et al.*, 1989). These results were supported in a subsequent study in which GH was selectively decreased to undetectable levels by neonatal administration of 4 mg MSG/g body weight to rats. The results showed an approximate doubling in adult male levels of CYP2A1 but only about a fifty percent increase in adult females (Waxman *et al.*, 1990). Figure 1.9. demonstrates the effect of neonatal gonadectomy, adult hypophysectomy, and neonatal administration of 4 mg MSG/g body weight on adult male and female rat hepatic CYP2A1 levels relative to that of normal adult male rats.

To determine the effect of the GH secretory pattern on levels of CYP2A1, GH was administered to hypophysectomized male and female rats both intermittently and by continuous infusion which led to suppression and induction, respectively, of CYP2A1. In summary, the rise in GH secretion characteristic of adult males appears to suppress the expression of CYP2A1 whereas the continuous level of plasma GH found in adult females is thought to maintain CYP2A1 at a higher level. Cytochrome P450 2A1



Figure 1.9. Effect of neonatal gonadectomy, adult hypophysectomy, and neonatal administration of 4 mg MSG/g body weight on adult male and female rat hepatic CYP2A1 levels relative to that of normal adult male rats. UT, untreated; Cx, neonatally castrated; Ox, neonatally ovariectomized; Hx, hypophysectomized; MSG, neonatally administered 4 mg MSG/g body weight (5 injections on alternate days during the first 9 days of life). (Waxman *et al.*, 1989; Waxman *et al.*, 1990)

1.7.2. Cytochromes P450 2B1 and 2B2

CYP2B1 and CYP2B2 share 97% similarity in their amino acid sequences (Waxman and Walsh, 1982). Together they constitute only 1 to 2% of total cytochrome P450 in untreated male and female rats but are highly inducible (approximately 20- to 30-fold increase) by phenobarbital. There is no sex difference in the expression of CYP2B1 and CYP2B2 in rats (Ryan and Levin, 1990), but the levels of both proteins are slightly higher in immature versus adult rats (Parkinson *et al.*, 1983a). Pentoxyresorufin O-dealkylase and testosterone 16 β -hydroxylase activity have been shown to reflect the hepatic level of CYP2B1 and CYP2B2 in rats treated with phenobarbital-type inducers (Sonderfan *et al.*, 2000).

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1987). Yamazoe *et al.* (1987) observed that hypophysectomy of adult male and female rats dramatically increased the levels of both CYP2B1 and CYP2B2 (Figure 1.10.), and this was reversed by either intermittent injection or continuous infusion of GH. These results indicate that GH suppresses the hepatic expression of CYP2B1 and CYP2B2 in the rat.



Figure 1.10. Effect of adult hypophysectomy on adult male and female rat hepatic CYP2B1/CYP2B2 levels relative to that of normal adult male rats. * indicates not determined, for abbreviations see Figure 1.9. (Yamazoe *et al.*, 1987)

1.7.3. Cytochrome P450 2C7

CYP2C7 is a constitutive female-predominant isozyme that is present at about 0.5% of total microsomal cytochrome P450 in rats at three weeks of age and increases to about 7% in adult males and 14 to 21% in adult females (Bandiera *et al.*, 1986; Bandiera and Dworschak, 1992). Bandiera and Dworschak (1992) investigated the effects of the sex steroids on the expression of CYP2C7. The level of CYP2C7 in adult male rats neonatally

castrated was similar to that of intact adult female rats (17% of total cytochrome P450) and this level was not significantly altered by administration of testosterone or estradiol during the neonatal, pubertal, or neonatal and pubertal periods. However, neonatally castrated male rats treated with testosterone during adulthood expressed CYP2C7 at the same level as intact. untreated adult males. Neonatally ovariectomized adult female rats displayed CYP2C7 at a level approximately half of the intact adult female rat level. This level was restored by administration of estradiol benzoate during adulthood or the pubertal period. Neonatally sham-operated adult female rats treated with testosterone or estradiol during any stage of development did not show any significant changes in CYP2C7 expression. The effect of GH on CYP2C7 has only been investigated in terms of mRNA expression but Sasamura et al. (1990) observed that the developmental changes in CYP2C7 mRNA were consistent with those observed in CYP2C7 protein (Bandiera et al., 1986), suggesting that CYP2C7 mRNA levels reflect CYP2C7 protein levels. Rats hypophysectomized at six or seven weeks of age displayed a dramatic decrease in hepatic CYP2C7 mRNA expression in both sexes but the sex difference remained approximately 2-fold (Westin et al., 1990; Sasamura et al., 1990). Treatment of hypophysectomized rats with intermittent injections of human GH restored hepatic CYP2C7 mRNA to a level similar to that observed in control male rats, while continuous infusion of human GH caused a greater increase in the mRNA to a level nearer to that observed in female control rats (Sasamura *et al.*, 1990). Catalytically, CYP2C7 displays retinol 4-hydroxylase activity (Leo and Lieber, 1985). Figure 1.11. demonstrates the effect of neonatal gonadectomy and adult hypophysectomy on adult male and female rat hepatic CYP2C7 mRNA levels relative to that of normal adult male rats.

Cytochrome P450 2C7



Figure 1.11. Effect of neonatal gonadectomy and adult hypophysectomy on adult male and female rat hepatic CYP2C7 levels relative to that of normal adult male rats. * indicates not determined, for abbreviations see Figure 1.9. (Westin *et al.*, 1990; Sasamura *et al.*, 1990; Bandiera and Dworschak, 1992)

1.7.4. Cytochrome P450 2C11

CYP2C11 is a male-specific isozyme that is associated with testosterone 2α -, 16α -, and 17-hydroxylase activity (Sonderfan *et al.*, 1987). Although it is undetectable in male rats less than four weeks old, levels of this isozyme increase during puberty to comprise a third of the total cytochrome P450 in males. The expression of CYP2C11 is suppressed in neonatally castrated adult male rats (Morgan *et al.*, 1985; Waxman *et al.*, 1985; Dannan *et al.*, 1986) but can be restored by testosterone treatment. It has not been detected in intact females at any age but neonatally ovariectomized rats treated with testosterone display CYP2C11 at a level similar to that of normal adult males (Dannan *et al.*, 1986). These studies indicate that CYP2C11 is testosterone dependent. Removal of GH, either through MSG-treatment (Shapiro *et al.*, 1989) or hypophysectomy, reduces CYP2C11 expression. Figure 1.12. displays the effect of neonatal gonadectomy, adult hypophysectomy, and neonatal administration of 4 mg MSG/g body weight on adult male and female rat hepatic CYP2C11 levels relative to that of normal adult male rats.



Cytochrome P450 2C11

Figure 1.12. Effect of neonatal gonadectomy, adult hypophysectomy, and neonatal administration of 4 mg MSG/g body weight on adult male and female rat hepatic CYP2C11 levels relative to that of normal adult male rats. For abbreviations see Figure 1.9. (Morgan *et al.*, 1985; Waxman *et al.*, 1985; Dannan *et al.*, 1986; Shapiro *et al.*, 1989; Waxman *et al.*, 1991)

Waxman *et al.* (1991) used an external pump to deliver pulses of rat GH of varying frequency and amplitude to hypophysectomized rats. They observed that CYP2C11 expression in hypophysectomized male rats was markedly stimulated by GH pulses administered at the characteristic male-like frequency of six times daily, as well as by the

uncharacteristic frequencies of two and four times daily. However, GH delivered at a frequency of seven times daily was ineffective or only marginally effective at stimulating CYP2C11 expression. From their results, Waxman *et al.* (1991) concluded that a critical minimum trough period of plasma GH is necessary for hepatic expression of CYP2C11.

1.7.5. Cytochrome P450 2E1

CYP2E1 is a female predominant isozyme in the rat (Waxman et al., 1989) that demonstrates *p*-nitrophenol hydroxylase (Reinke and Moyer, 1985; Koop, 1986; Tassaneeyakul et al., 1993), N-nitrosodimethylamine demethylase (Miller and Yang, 1984; Levin et al., 1986), aniline hydroxylase (Morgan et al., 1982) and paracetamol hydroxylase (Morgan et al., 1983; Raucy et al., 1989) activities. It is present in rat liver at a level that is 1.5- to 2-fold higher in adult female rats than their male counterparts. CYP2E1 is induced by treatment with ethanol, acetone, pyrazole, isoniazid, or dimethyl sulfoxide, as well as by diabetes or starvation (Koop and Tierney, 1990). Its highest level of expression is during the late suckling period, after which it is suppressed in both sexes, but to a greater extent in males than in females (Thomas et al., 1987). Male and female adult rats that were gonadectomized neonatally or during adulthood were found to have a significant increase in CYP2E1 levels (Waxman et al., 1989). This increase in the level of expression was reversed by the administration of androgen or estradiol, indicating that both of these sex hormones suppress CYP2E1. Due to the influence of the GH secretory pattern on hepatic cytochrome P450 expression, hypophysectomy studies were performed. Both male and female adult hypophysectomized rats demonstrated an increase in CYP2E1 to prepubertal levels that was largely reversed by GH administered by either intermittent injection or continuous infusion. Similarly, depletion of serum GH by MSG treatment resulted in a 2-fold increase in CYP2E1 levels in both sexes (Waxman *ét al.*, 1990). These results suggest that GH has a suppressive effect on the expression of CYP2E1. Figure 1.13. demonstrates the effect of neonatal gonadectomy, adult hypophysectomy, and neonatal administration of 4 mg MSG/g body weight on adult male and female rat hepatic CYP2E1 levels relative to that of normal adult male rats.



Figure 1.13. Effect of neonatal gonadectomy, adult hypophysectomy, and neonatal administration of 4 mg MSG/g body weight on adult male and female rat hepatic CYP2E1 levels relative to that of normal adult male rats. For abbreviations see Figure 1.9. (Waxman *et al.*, 1989; Waxman *et al.*, 1990)

1.7.6. Cytochrome P450 3A1

CYP3A1 is not detectable in untreated male or female rats of any age (Cooper et al.,

1993). However, this isozyme is inducible by pregnenolone- 16α -carbonitrile,

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dexamethasone, and the macrolide antibiotic, triacetyloleandomycin, up to a level of approximately 37% of adult total cytochrome P450 (Cooper *et al.*, 1993). All members of the CYP3A family identified in the rat to date hydroxylate testosterone at the 2β -, 6β - and 15β -positions (Waxman *et al.*, 1985; Sonderfan *et al.*, 1987; Nagata *et al.*, 1990).

1.7.7. *Cytochrome P450 3A2*

CYP3A2 and its associated testosterone 6β-hydroxylase activity are present in untreated immature rats of both sexes and in untreated adult males but they have not been detected in untreated adult females (Cooper et al., 1993). Waxman et al. (1988) reported that castration of male rats at birth markedly reduced the expression of CYP3A2 in adult male rats whereas birth ovariectomy did not cause a major expression of this isozyme in adult female rats. Hypophysectomy studies have indicated that CYP3A2 is suppressed by GH (Waxman et al., 1988). Male and female hypophysectomized rats demonstrate about a 2-fold and 10- to 20-fold increase, respectively, in CYP3A2 expression. When GH is administered to hypophysectomized male and female rats in their characteristic secretory pattern, the females showed a dramatic decrease in the level of this isozyme back towards the normal level, while the males also demonstrated a decrease but not as pronounced (Waxman et al., 1988). In contrast to these studies, depletion of GH in rats by neonatal MSG administration did not result in elevated levels of CYP3A2 in adult males (Shapiro et al., 1989) or in the expression of this isozyme in adult females (Waxman et al., 1990). Thus, expression of CYP3A2 is affected by factors other than GH levels alone. Figure 1.14. shows the effect of neonatal gonadectomy, adult hypophysectomy, and neonatal administration of 4 mg MSG/g

body weight on male and female rat hepatic CYP3A2 levels relative to that of normal adult male rats.



Figure 1.14. Effect of neonatal gonadectomy, adult hypophysectomy, and neonatal administration of 4 mg MSG/g body weight on male and female rat hepatic CYP3A2 levels relative to that of normal adult male rats. For abbreviations see Figure 1.9. (Cooper *et al.*, 1993; Waxman *et al.*, 1988; Shapiro *et al.*, 1989; Waxman *et al.*, 1990)

1.8. INDUCTION OF CYTOCHROMES P450 BY TAMOXIFEN

Recently a study was published that demonstrated the direct effect of large acute doses of tamoxifen on specific cytochrome P450 isozymes. White *et al.* (1992) administered tamoxifen to rats at a dose of 45 mg/kg/day for four days to investigate its effects on hepatic microsomal cytochromes P450 and monooxygenase activities. The dose of tamoxifen used in their study (45 mg/kg/day) is dramatically higher than that used by women (20 mg/day) but women are usually on tamoxifen therapy for many years. Their study found that the

metabolism of pentoxy- and benzyloxyresorufin was induced 30- to 60-fold while ethoxyresorufin O-deethylase activity was only slightly increased in hepatic microsomes prepared from female rats. The level of induction was higher when tamoxifen was administered by oral gavage (*p.o.*) instead of intraperitoneally (*i.p.*). The extent of induction was also dependent on the dose administered and duration of exposure to tamoxifen. For example, testosterone hydroxylation at the 6 β - and 16 α -positions was induced in a dosedependent manner up to 0.12 mmol/kg (45 mg/kg) and maximal induction occurred between three and seven days after dosing. The lowest dose tested, 13 µmol/kg (5 mg/kg) *i.p.* for four days, still caused significant increases in testosterone 6 β -hydroxylation and androstenedione formation. Immunoblots demonstrated that tamoxifen pretreatment (0.12 mmol/kg *p.o.* for four days) modestly increased the concentrations of CYP2B1, CYP2B2 and CYP3A proteins 2- to 3-fold in rat hepatic microsomes. On the basis of the results from White's investigation, it appears that tamoxifen has a direct inductive effect on CYP2B1, CYP2B2 and CYP3A.

Tannenbaum *et al.* (1992), however, have reported that there was no significant alteration of the GH secretory pattern in female rats 18 hours after tamoxifen treatment and only slight attenuation of GH levels one week post-administration. Pronounced suppression did not occur until three to seven weeks after treatment. Thus, the hormonally-mediated effects of tamoxifen on cytochromes P450 have not been investigated previously.

-1.9. OBJECTIVES OF THE PRESENT INVESTIGATION

The overall goal of this investigation was to characterize the effects of tamoxifen, when administered at a dose and regimen which had previously been shown to have inhibitory effects on DMBA-induced mammary tumors and to profoundly suppress GH secretion in rats, on the hepatic expression of cytochromes P450. The specific objectives of this study were:

(1) To examine the effects of tamoxifen administration on body weight, liver weight and serum estradiol levels,

(2) To investigate the effects of tamoxifen administration on total hepatic cytochrome P450 content and hepatic microsomal enzyme activities including testosterone 2α -, 6β -, 7α -, 16α -, and 16β -hydroxylase activities, *p*-nitrophenol hydroxylase activity and pentoxyresorufin O-depentylase activity,

(3) To determine the effects of tamoxifen on individual hepatic microsomal cytochrome P450 protein levels including CYP2A1, CYP2C7, CYP2E1 and CYP3A, and

(4) To compare the effects of tamoxifen treatment with the effects of neonatal ovariectomy, with or without subsequent treatment with estradiol during adulthood, on cytochrome P450 expression with the aim of gaining insight into the mechanism of action of tamoxifen.

Regulation of these enzymes is extremely complex, with any single factor leading to preferential increases in one or more isozymes while having a repressive effect on the levels of other isozymes. Changes in hepatic levels of individual cytochromes P450 can lead to an altered ability of the liver to metabolize foreign and physiologic compounds, which can result in a shift in steroid hormone levels, an increased or decreased response to drugs, and an altered susceptibility to potentially toxic and carcinogenic xenobiotics. Thus, it is important to have a complete understanding of which factors alter the expression and/or catalytic activity of individual cytochromes P450 so that the induction or inhibition of the activity of specific isozymes on their substrates can be predicted.

2. EXPERIMENTAL

2.1. CHEMICALS

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

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

Resorufin and trisodium citrate dihydrate.

Anachemia (Mississauga, Ontario, Canada):

Potassium phosphate.

BDH Chemicals (Toronto, Ontario, Canada):

Ascorbic acid, citric acid (anhydrous), Coomassie Brilliant Blue 250, disodium hydrogen orthophosphate (dibasic), ethanol (95%), ethylenediaminetetraacetic acid disodium salt (EDTA), glycine, magnesium chloride, methanol, potassium dihydrogen orthophosphate (monobasic), potassium chloride, propylene glycol, sodium chloride, sodium hydroxide, sucrose, and sulphuric acid.

Bio-Rad Laboratories (Hercules, California, U.S.A.):

Amplified Alkaline Phosphatase Immun-Blot Assay kit (*p*-nitroblue tetrazolium chloride (NBT) in 70% dimethylformamide and 5-bromo-4-chloro-3-indolylphosphate (BCIP) in dimethylformamide).

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

Acrylamide 99.9%, ammonium persulphate 98%, N,N'-methylene-bis-acrylamide (BIS), 2-mercaptoethanol, sodium dodecyl sulphate (SDS), and N,N,N',N'-tetramethyethylenediamine (TEMED).

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

Nicotinamide adenine dinucleotide phosphate tetra-sodium salt reduced form (NADPH).

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

Skim milk powder.

Chemonics Scientific (Anachemia Canada Inc., Richmond, B.C., Canada):

Glycerol, hydrochloric acid, and hydrogen peroxide 30% solution.

Fisher Scientific Ltd. (Vancouver, B.C., Canada):

Acetonitrile (Optima grade), bromphenol blue (tetrabromophenol sulfon phthalein), methanol (HPLC grade), methylene chloride (HPLC grade), perchloric acid 70%, sodium bicarbonate, and sodium carbonate. GIBCO-BRL Canada (Burlington, Ontario, Canada):

Goat serum (heat inactivated by incubating at 56°C for 30 minutes and then cooling on ice), and Nunc immunoplates.

Hershey Canada Inc. (Mississauga, Ontario, Canada):

Peanut oil (100% pure).

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

Bovine serum albumin, globulin and fatty acid free, fraction V (BSA), sodium cholate, and RSL Direct ^{125}I Estradiol 17 β kit.

Immunocorp (Montreal, Quebec, Canada):

Affinity isolated goat $F(ab')_2$ anti-(rabbit immunoglobulins) gamma and light chains human Ig adsorbed peroxidase conjugate (TAGO Immunological).

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

Sodium dithionite powder (purified), and sodium phosphate.

Kao Corporation Chemicals Division (Tokyo, Japan):

Emulgen 911.

Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, Maryland, U.S.A.):

Affinity purified antibody peroxidase labeled rabbit anti-sheep IgG (γ), and affinity purified antibody phosphatase labeled rabbit anti-sheep IgG (H+L).

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

Blotting paper and nitrocellulose membrane (Schleicher & Schuell).

Medigas Pacific (Vancouver, B.C., Canada):

Carbon monoxide gas (99.5% purity).

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

7-pentoxyresorufin.

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

Cupric sulfate, β -estradiol 3-benzoate, Folin and Ciocalteu's phenol reagent, HEPES, 4-nitrocatechol (1,2-dihydroxy-4-nitrobenzene), *p*-nitrophenol (spectrophotometric grade), *o*-phenylenediamine (free base), Pyronin Y (certified), sodium/potassium tartrate tetrahydrate, tamoxifen ([Z]-1-[p-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene), Trizma (Tris) base, Trizma HCl, and Tween 20.

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

4-androsten-17β-diol-3-one (testosterone), 4-androsten-3,17β-diol-3-one (androstenedione), 4-androsten-2 α ,17β-diol-3-one (2 α -hydroxytestosterone), 4-androsten-6 β ,17β-diol-3-one, 4-androsten-7 α ,17β-diol-3-one, 4-androsten-11 β ,17β-diol-3-one, 4-androsten-16 α ,17 β -diol-3-one, and 4-androsten-16 β ,17 β -diol-3-one.

TAGO, Inc. (Burlingame, California, U.S.A.):

Goat $F(ab')_2$ anti-(rabbit IgG) (gamma and light chain specific) affinity isolated antibodies alkaline phosphatase conjugated and affinity isolated goat $F(ab')_2$ anti-(mouse immunoglobulins) gamma and light chains human Ig adsorbed peroxidase conjugate.

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

Purified rat CYP2C7 and CYP2C11 and partially purified rat CYP3A1.

Monospecific polyclonal rabbit anti-CYP2C7 IgG, rabbit anti-CYP2C11 IgG, and rabbit anti-CYP3A1 IgG (backabsorbed).

Dr. A. Parkinson (University of Kansas Medical Center, Kansas City, Kansas, U.S.A.): Purified CYP2A1. Dr. P.E. Thomas (Department of Chemical Biology and Pharmacognosy, The State University of New Jersey, Rutgers, Piscataway, New Jersey, U.S.A.):

Sheep anti-CYP2A1 polyclonal IgG, rabbit anti-CYP2E1 polyclonal IgG, mouse anti-CYP3A1 monoclonal IgG, and mouse anti-CYP3A2 monoclonal IgG.

2.2. ANIMALS AND TREATMENT

Adult male (237-275 g) and female (199-240 g) Long Evans rats were obtained from Charles River Canada, Inc. (Montreal, Quebec, Canada). The animals were housed in polycarbonate cages on pesticide-free corncob bedding, under conditions of controlled temperature (23°C) and 12-h light and 12-h dark cycles. Food (Ralston-Purina Certified Rodent Chow 5001) and tap water were available *ad libitum*. After one week of acclimatization, rats were injected subcutaneously with 5 mg tamoxifen in 0.2 mL peanut oil once daily for 2 consecutive days (Jordan, 1976). Control rats received 0.2 mL peanut oil subcutaneously at the same time points. This dosing regimen with tamoxifen had previously been shown to dramatically suppress both the amplitude of spontaneous GH secretory bursts and the mean 6-h plasma GH levels from 3 to 7 weeks post-treatment in both sexes as compared to their controls (Tannenbaum *et al.*, 1992). Rats were sacrificed by decapitation 5 weeks after treatment.

Pregnant Long Evans rats at 15 days of gestation were purchased from Charles River Canada Inc. and maintained under the same conditions as described above. Newborn female pups were either ovariectomized or sham-operated under halothane anaesthesia within 24 hours of birth. One group of neonatally ovariectomized and one group of sham-operated rats received no further treatment, while a second group was injected subcutaneously with

estradiol benzoate (0.5 mg/kg/injection dissolved at 0.5 mg/mL in propylene glycol) during adulthood beginning at 8 weeks of age and continuing for 14 days, as described previously (Bandiera and Dworschak, 1992). Neonatally ovariectomized and sham-operated rats were kept at normal controlled conditions and sacrificed by decapitation at 10 weeks of age.

2.3. PREPARATION OF HEPATIC MICROSOMAL FRACTION

Hepatic microsomes were prepared from individual rats immediately following decapitation using the method of Thomas *et al.* (1983). Livers were quickly removed, weighed, minced, placed in 20 mL of ice-cold 0.05 M Tris, 1.15% KCl, pH 7.5, and homogenized using a Potter-Elvehjem glass mortar and a motor-driven pestle by 5 slow-speed passes with a loose-fitting pestle and 5 high-speed passes with a tight-fitting pestle. The homogenate was centrifuged at 9,000 x g for 20 min at 5°C in a Beckman centrifuge and the resulting supernatant was centrifuged at 105,000 x g for 60 min at 5°C. The glycogen was discarded and the resulting microsomal pellets were resuspended in ice-cold 10 mM EDTA, 1.15% KCl, pH 7.4, using the homogenizer with 5 passes of the loose-fitting pestle, and centrifuged again at 105,000 x g for 60 min at 5°C. The pellets were resuspended in 0.25 M sucrose by gentle homogenization and then diluted in 0.1 M sodium phosphate, pH 7.4, 20% glycerol, 0.1 mM EDTA to give a final microsomal protein concentration of 10-30 mg/mL. Aliquots were stored at -80°C for subsequent assays.

2.4. DETERMINATION OF TOTAL CYTOCHROME P450 CONTENT

Total hepatic microsomal cytochrome P450 was measured spectrophotometrically as described by Omura and Sato (1964a). Hepatic microsomes were reduced with sodium dithionite and then saturated with carbon monoxide. The carbon monoxide-reduced difference spectrum was measured on a SLM-Aminco DW-2 spectrophotometer and determined as the difference between the absorption maximum at 450 nm and the baseline at 490 nm. Total hepatic cytochrome P450 concentration was calculated using a molar extinction coefficient of 91 cm²/mmol (Omura and Sato, 1964b).

2.5. DETERMINATION OF PROTEIN CONTENT

Total hepatic microsomal protein was measured by the method of Lowry *et al.* (1951). Bovine serum albumin was used as the standard. All samples were measured in duplicate at an absorbance of 720 nm.

2.6. SERUM PREPARATION

Blood was collected at the time of sacrifice to enable the measurement of estradiol levels subsequently. The blood was incubated at 37°C for 1 h and then kept at 4°C overnight. To separate the serum, the blood was centrifuged at 2,000 rpm for 10 min. The serum was stored at -20°C until needed.

2.7. MEASUREMENT OF SERUM ESTRADIOL CONCENTRATION

Total unconjugated 17 β -estradiol was measured in the serum collected at the time of sacrifice using an ¹²⁵I radioimmunoassay kit (ICN Biomedicals, Inc.). The samples were measured in duplicate and a standard curve was constructed simultaneously to determine the estradiol concentration. The detection limit of the assay was reported by the manufacturer as 10 pg/mL.

2.8. ENZYME ASSAYS

2.8.1. Testosterone hydroxylase assay

The formation of monohydroxylated testosterone metabolites was determined based on the method of Sonderfan *et al.* (1987). The cytochrome P450 catalyzed hydroxylation of testosterone to the metabolites monitored in this study is shown below:



Scheme 1. Illustration of the positions of testosterone 2α -, 6β -, 7α -, 16α - and 16β -hydroxylation.

Testosterone (0.25 mM) was incubated for 5 min at 37°C in 1-mL incubation mixtures containing potassium phosphate buffer (46 mM, pH 7.4), MgCl₂ (2.7 mM), NADPH (1.0 mM), and liver microsomes (0.3 nmol cytochrome P450), at the final concentrations indicated. Reactions were started by addition of the testosterone and stopped by addition of 6 mL of methylene chloride. Each sample was spiked with 2.5 nmol of 11 β -hydroxytestosterone (in methanol) and shaken vigorously for 2 min. The two phases were

separated by centrifugation (1000 x g for 1 min), the aqueous phase was aspirated and discarded, and the organic phase was evaporated under a stream of nitrogen. The residue was redissolved in 200 µL methanol with vortex mixing for 1 min. A 10 µL aliquot was analyzed by HPLC. The monohydroxylated testosterone isomers, 2α -, 6β -, 7α -, 16α -, and 16β hydroxytestosterone, were resolved at 40° C on a reverse-phase C₁₈ column with a Shimadzu LC-6A binary gradient HPLC system equipped with an SIL-6B auto injector (Shimadzu Scientific Instruments). A Supelcosil reverse-phase octyldecylsilane column (5 μ m, 15 cm \times 4.6 mm i.d.) was used and was preceded by a 2 cm LC-18 guard column (40 µm) (Supelco, Bellefonte, PA). A concave gradient from 90% solvent A (methanol:water:acetonitrile, 39:60:1) to 85% solvent B (methanol:water:acetonitrile, 80:18:2) was operated over 40 min at 1.5 mL/min to elute testosterone and its potential metabolites. A variable wavelength UV detector (Shimadzu SPD-6A) was used to monitor the metabolites at 254 nm. The metabolites were identified by comparing retention times to those of authentic standards. The quantity of each metabolite formed was determined from the slope of a linear calibration curve using the ratio of the peak area (integrated by a Shimadzu CR501 chromatography data processor) of the metabolite to that of the internal standard. Calibration curves were generated for each metabolite by plotting the ratio of the peak area of the authentic standard to that of the internal standard versus the concentration of the authentic standard. Calibration curves were prepared as part of every assay and used four concentrations of each authentic standard. Tubes to which authentic standards were added contained the complete reaction mixture except that the addition of the testosterone was replaced by the addition of the authentic standard mixture.

2.8.2. *p*-Nitrophenol hydroxylase assay

The cytochrome P450 catalyzed hydroxylation of *p*-nitrophenol is shown below:



4-nitrophenol

4-nitrocatechol

Scheme 2. Illustration of *p*-nitrophenol hydroxylation.

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol (2,4-dihydroxy-4-nitrobenzene) was measured using the method of Koop (1986). *p*-Nitrophenol (100 μ M) was incubated for 5 min at 37°C in 2-mL incubation mixtures containing potassium phosphate buffer (0.1 M, pH 6.8), MgCl₂ (5 mM), ascorbic acid (1 mM), NADPH (0.5 mM), and liver microsomal protein (2 mg/mL), at the final concentrations indicated. Reactions were initiated with NADPH after preincubation for 10 min at room temperature and were terminated by addition of 1 mL of 0.6 M perchloric acid followed immediately by vortex mixing. The tubes were spun in a centrifuge at 2,000 x g for 10 min at room temperature. The supernatant was removed and 0.2 mL of 10 M NaOH solution was added to 2.5 mL of the clear supernatant, mixed on a vortex, and the absorbance was read at 546 nm on a Shimadzu spectrophotometer. The amount of 4-nitrocatechol present was determined from a calibration curve.

2.8.3. Pentoxyresorufin O-depentylase assay

The cytochrome P450 catalyzed depentylation of pentoxyresorufin is illustrated below:



Scheme 3. Illustration of pentoxyresorufin O-depentylation.

The depentylation of pentoxyresorufin to resorufin was determined based on the method of Lubet *et al.* (1985). Pentoxyresorufin (5 μ M) was incubated for 5 min at 37°C in 2-mL incubation mixtures containing HEPES buffer (97 mM, pH 7.8), MgCl₂ (4.8 mM), NADPH (0.25 mM), and liver microsomal protein (0.2 mg/mL), at the final concentrations indicated. Reactions were initiated with NADPH after preincubation for 5 min at 37°C. The reaction was allowed to proceed for 5 min. The fluorescence intensity was recorded at zero time before the addition of NADPH and after 5 min of incubation. The amount of resorufin formed was calculated from a calibration curve.

2.9. IMMUNOQUANTITATION OF CYTOCHROME P450 PROTEIN LEVELS

2.9.1. Competitive ELISA

Quantitative determinations of CYP2A1, CYP2C7 and CYP3A in liver microsomes were attempted by competitive ELISA. The coating antigen (highly purified CYP2A1, CYP2C7 or CYP3A) was diluted to 0.2 μ g/mL in 0.1 M sodium carbonate-bicarbonate

buffer, pH 9.5 and 150 μ L was added to each well (except for the first column) of a 96-well microtest plate (Nunc, Vanguard International Inc., Neptune, N.J.). As a negative control the buffer alone was added to each well of the first column. The plates were covered and incubated at 4°C overnight. Meanwhile, solutions of competing antigens were prepared as follows. Hepatic microsomes were first solubilized by mixing equal volumes of microsomes and solubilization buffer (0.1 M KPO₄, pH 7.4, 0.1 mM EDTA, 20% glycerol, 0.5% sodium cholate, 0.2% Emulgen 911) and then serially diluted to the appropriate concentrations in antibody dilution buffer (0.1% BSA, 20% heat inactivated normal goat serum, in modified PBS) containing 0.25% sodium cholate, 0.1% Emulgen 911, and monospecific primary antibody (rabbit anti-CYP). A standard curve of purified CYP2A1, CYP2C7 or CYP3A was also prepared by using the same buffers as above. The various dilutions of cytochrome P450 and hepatic microsomes were incubated overnight at 4°C.

The following morning the coated plates were emptied and blocked with a solution of 0.3% BSA in PBS at 37°C for 1 h. The plates were washed three times (for about 2 min each) with wash buffer (0.05% Tween 20 in modified PBS) to remove unbound protein. Next, 150 μ L of the competing antigen solutions were added to each coated well. In a typical assay, eight concentrations each of four different microsomal samples and either CYP2A1, CYP2C7 or CYP3A were tested per coated 96-well microtest plate. The plates were incubated at 37°C for 3 h with gentle shaking. The plates were washed three times as described above to remove unbound antibody. Horseradish peroxidase-conjugated secondary antibody was diluted in antibody dilution buffer and 150 μ L was added to each well. The plates were washed three times as described at 37°C for 1 h with gentle shaking. The plates were washed three times as described at 37°C for 1 h with gentle shaking.

times as described above to remove excess conjugated antibody. The amount of conjugated antibody bound in each well was quantitated by reaction for 10 min with 150 μ L of freshly prepared chromagen substrate solution (2.2 mM *o*-phenylenediamine, 0.01% H₂O₂ in 0.1 M sodium citrate buffer, pH 5.0). The plates were incubated at room temperature and the reaction was stopped with the addition of 40 μ L of 8 N H₂SO₄. The absorbance through each well was read at 490 nm using an Automated Microplate Reader Model EL309 (Bio-Tek Instruments Inc. Laboratory Division).

2.9.2. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) using a Hoefer SE 600 vertical slab gel unit. The discontinuous SDS-polyacrylamide gel consisted of a 7.5% acrylamide separating gel (0.75 mm thick, 12.5 cm long) and a 3.0% stacking gel (0.75 mm thick, 1.0 cm long). The separating gel contained 0.375 M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 7.5% (w/v) acrylamide-BIS, 0.042% (w/v) ammonium persulphate, and 0.03% (v/v) TEMED and the stacking gel contained 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 3.0% (w/v) acrylamide-BIS, 0.08% (w/v) ammonium persulphate, and 0.05% (v/v) TEMED. Protein was denatured by boiling the microsomal samples for 2 min in sample dilution buffer containing 0.062 M Tris-HCl buffer (pH 6.8), 1% (w/v) SDS, 10% glycerol, 0.001% (w/v) bromophenol blue, and 5% (v/v) mercaptoethanol. Microsomal proteins (10 µg/well) were subjected to electrophoresis at a constant current of 0.12 mA per gel through the stacking gel

and 0.24 mA per gel through the separating gel. Individual cytochromes P450 resolved by SDS-PAGE were identified using immunoblots.

2.9.3. Immunoblots

Individual cytochromes P450 were detected immunochemically using the method of Towbin et al. (1979). After the proteins were separated by SDS-PAGE they were electrophoretically transferred onto a 13 x 14 cm sheet of nitrocellulose using a Hoefer Transphor Apparatus (Model TE 52) at a setting of 0.4 A for 2 h at 4°C. The nonspecific antibody binding sites were blocked by incubating the nitrocellulose membranes in blocking buffer (1% BSA, 3% skim milk powder, in modified PBS) overnight at 4°C. The next morning the blocking buffer was discarded and the nitrocellulose membranes were incubated with the primary antibody diluted appropriately in antibody dilution buffer (1% BSA, 3% skim milk powder, 0.05% Tween 20, in modified PBS) at 37°C with shaking for 2 h. The membranes were washed three times in wash buffer (0.05% Tween 20 in modified PBS) where each wash cycle consisted of a 10 min incubation at 37°C in 50 mL of wash buffer with shaking. The nitrocellulose membranes were incubated with the secondary antibody diluted appropriately in antibody dilution buffer at 37°C with shaking for 2 h. The membranes were washed three times as described above and allowed to completely drain. The substrate solution (0.01% NBT, 0.005% BCIP, in 0.1 M Tris-HCl buffer, 5mM MgCl₂, pH 9.5) was added to the nitrocellulose membranes under subdued light. After the appropriate length of time the reaction was stopped by immersing the membranes in distilled water.

For immunodetection of CYP2A1, the primary antibody (sheep anti-CYP2A1 polyclonal IgG) was used at a concentration of 40 µg/mL, the secondary antibody (rabbit anti-sheep IgG) was used at a dilution of 1:250, and the phosphatase reaction was terminated after 7 min. For immunodetection of CYP2C7, the primary antibody (rabbit anti-CYP2C7 polyclonal IgG) was used at a concentration of 100 µg/mL, the secondary antibody (goat anti-rabbit IgG) was used at a dilution of 1:1000, and the phosphatase reaction was terminated after 6.5 min. For immunodetection of CYP2E1, the primary antibody (rabbit anti-CYP2E1 polyclonal IgG) was used at a concentration of 10 µg/mL, the secondary antibody (goat anti-rabbit IgG) was used at a dilution of 1:3000, and the phosphatase reaction was terminated after 5 min. For immunodetection of CYP3A, the primary antibody (rabbit anti-CYP3A polyclonal IgG) was used at a concentration of 50 ug/mL, the secondary antibody (goat anti-rabbit IgG) was used at a dilution of 1:3000, and the phosphatase reaction was terminated after 7 min. For immunodetection of CYP3A1 and CYP3A2 using monoclonal antibodies, the primary antibodies (mouse anti-CYP3A1 and mouse anti-CYP3A2 monoclonal IgG) were used at a concentration of 0.25 µg/mL, the secondary antibody (goat anti-mouse IgG) was used at a dilution of 1:3000, and the peroxidase reaction was terminated after 7.5 min.

2.9.4. Densitometry

The staining intensity of the bands on the nitrocellulose membranes was measured using computer image analysis with a VISAGE 110 Bio Image Analyzer (Bio Image, Ann Arbor, Michigan) consisting of a high resolution camera and a Sun Microsystems Workstation. The amount of immunoreative protein present in each lane was determined from the integral of the optical density of the stained band using the software option Whole Band Analysis. Each microsomal sample was applied to each gel at a constant protein concentration of 10 μ g/well.

For quantitation of CYP2A1 and CYP3A, densitometry values were converted into pmole quantities using calibration curves. Calibration curves were generated by loading 0.03125, 0.0625, 0.125, 0.250, 0.375 and 0.500 pmol of purified rat CYP2A1 or CYP3A per lane. Also, a single concentration of purified cytochrome P450 standard was included on each blot as an internal standard. Conditions for electrophoresis and immunoblotting were as described above.

2.10. STATISTICAL ANALYSIS

All data are presented as the mean \pm standard error of the mean of values obtained from individual rats. The mean values of the tamoxifen-treated group were compared to those of the vehicle-treated group using the unpaired Student's *t*-test (GraphPAD InStat, 1990). Mean values for adult female rats that were either neonatally ovariectomized, neonatally sham-operated, neonatally ovariectomized and administered estradiol during adulthood, or neonatally sham-operated and administered estradiol during adulthood were compared using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post-hoc test (Systat Version 5.01, 1990). Results were considered to be significant if p<0.05.
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3. RESULTS

In the present study, the dosing regimen used by Tannenbaum et al. (1992) was followed. Accordingly, adult male and female rats were administered 5 mg of tamoxifen subcutaneously once daily for two consecutive days while controls were given the same volume of vehicle (peanut oil) only. As GH levels in both adult male and female rats were reported to be dramatically reduced from three to seven weeks post-treatment, the animals in this study were sacrificed five weeks after tamoxifen treatment to ensure that their GH levels would be similar to the values measured in Tannenbaum's study. The effect of neonatal ovariectomy on cytochrome P450 expression was also investigated for the purpose of comparison and to gain some insight into the mechanism of action of tamoxifen. Microsomes were prepared previously from adult female rats that had been either neonatally ovariectomized, neonatally sham-operated, neonatally ovariectomized and administered estradiol during adulthood, or neonatally sham-operated and administered estradiol during adulthood (Bandiera and Dworschak, 1992). Analysis of the results from these groups should allow us to investigate the influence of estrogen on the catalytic activities and protein levels of specific cytochrome P450 isozymes.

Data for both male and female rats are presented in the first few sections of the Results. The reason for including data from male rats is to demonstrate the effects of tamoxifen on various parameters for two reasons: i) to ensure that tamoxifen is producing an effect and ii) to determine if these effects are sex specific.

3.1.a. EFFECT OF TAMOXIFEN ON BODY WEIGHT AND LIVER WEIGHT

All rats in the tamoxifen study were weighed on the first day of injection and at the time of sacrifice five weeks later. Mean values of body weight and liver weight for tamoxifen-treated and vehicle-treated male and female rats are presented in Table 3.1.a. Both the male and female rats treated with tamoxifen weighed significantly (p<0.05) less than their respective controls. The decrease in growth rate in the tamoxifen-treated rats supports the finding by Tannenbaum *et al.* (1992) that the dosing regimen used reduced GH levels. The liver weights of the tamoxifen-treated rats were also significantly less than those of the vehicle-treated controls but the liver weight as percent body weight did not change with tamoxifen treatment.

Sex	Treatment ^a	Final Body Weight (g)	% Change in Body Weight Over 5 Weeks	Liver Weight (g)	Liver Weight as % Body Weight
Male (n=8)	Vehicle	464±13	+78%	20.2±1.2	4.4±0.3
Male (n=9)	Tamoxifen	343±8†	+37%	15.0±0.5†	4.4±0.2
Female(n=8)	Vehicle	289±6	+28%	10.3±0.2	3.6±0.1
Female(n=9)	Tamoxifen	243±7†	+15%	8.7±0.4†	3.6±0.1

Table 3.1.a. Effect of tamoxifen treatment on body and liver weights in adult male and female rats.

Each value in the mean \pm standard error of the mean.

^a Rats were treated at 8 weeks of age and sacrificed at 13 weeks of age.

[†] Mean value of the tamoxifen-treated group is statistically different (p<0.05) from that of the vehicle-treated group.

3.1.b. EFFECT OF NEONATAL OVARIECTOMY WITH AND WITHOUT ADMINISTRATION OF ESTRADIOL DURING ADULTHOOD ON BODY WEIGHT AND LIVER WEIGHT

Mean body and liver weights of neonatally ovariectomized adult female rats (see Table 3.1.b.) did not vary significantly from those of their sham-operated counterparts. However, treatment of both ovariectomized and sham-operated females with estradiol during adulthood resulted in a small apparent increase in liver weight that was significant when expressed as a percent of body weight compared to rats that were not administered estradiol during adulthood.

Sex	Treatment ^a	Final Body Weight (g)	% Change in Body Weight Over 5 Weeks	Liver Weight (g)	Liver Weight as % Body Weight
Female(n=6)	Sham	231±10	NA	9.9±0.7	4.3±0.3
Female(n=9)	Ovariectomy	256±10	NA	10.7±0.5	4.2±0.2
Female(n=6)	Sham + Estradiol	215±8	NA	11.7±0.9	5.4±0.4‡
Female(n=6)	Ovariectomy + Estradiol	228±5	NA	12.8±0.8	5.6±0.3‡

Table 3.1.b. Effect of neonatal ovariectomy with and without administration of estradiol during adulthood on body and liver weights in adult female rats.

Each value in the mean \pm standard error of the mean.

^a Rats were either neonatally sham-operated (sham) or ovariectomized. The estradiol treated rats received daily subcutaneous injections of estradiol benzoate from 8 to 10 weeks of age. All rats were sacrificed at 10 weeks of age.

NA indicates not applicable.

^{\ddagger} Mean value of the estradiol-treated group is statistically different (p<0.05) from that of the appropriate group not administered estradiol.

In comparison with tamoxifen-treated female rats, mean body weights of the neonatally ovariectomized and sham-operated female rats were less than those of the vehicle-treated female rats in the tamoxifen study, but the latter rats were three weeks older at the time of death. Liver weights of the neonatally ovariectomized and sham-operated female rats were similar to those of the vehicle-treated female rats in the tamoxifen study, but liver weight as percent body weight was slightly greater for the neonatally ovariectomized and sham-operated rats as compared to the vehicle-treated female rats.

3.2.a. EFFECT OF TAMOXIFEN ON SERUM ESTRADIOL LEVELS

Individual serum estradiol levels were measured in blood samples collected at the time of sacrifice using an ¹²⁵I-radioimmunoassay. Serum concentrations of estradiol in tamoxifen-treated and vehicle-treated male and female rats are shown in Table 3.2.a. The mean serum estradiol levels did not vary significantly between tamoxifen-treated female rats and their respective controls.

3.2.b. EFFECT OF NEONATAL OVARIECTOMY WITH AND WITHOUT ADMINISTRATION OF ESTRADIOL DURING ADULTHOOD ON SERUM ESTRADIOL LEVELS

Serum estradiol concentrations were also determined for neonatally ovariectomized and sham-operated female rats (Table 3.2.b.). The neonatally sham-operated rats had an estradiol level near the top of the range reported by Butcher *et al.* (1974) for normal adult female rats. As expected, rats that were given estradiol during adulthood had significantly greater estradiol levels than the rats not administered estradiol.

Sex	Treatment ^a	Serum Estradiol Level (pg/mL)
Male (n=8)	Vehicle	ND
Male (n=9)	Tamoxifen	ND
Female (n=8)	Vehicle	168±25
Female (n=9)	Tamoxifen	124±9

Table 3.2.a. Effect of tamoxifen treatment on serum estradiol levels in adult rats.

Each value is the mean \pm standard error of the mean.

^a Rats were treated at 8 weeks of age and sacrificed at 13 weeks of age.

ND indicates not determined.

Table 3.2.b.	Effect of	neonatal	ovariectomy	with	and	without	administration	of	estradiol
during adultho	ood on seru	um estradi	iol levels in a	dult fe	emal	e rats.			

Sex	Treatment ^a	Serum Estradiol Level (pg/mL)
Female (n=6)	Sham	76±5
Female (n=9)	Ovariectomy	95±8
Female (n=6)	Sham + Estradiol	1403±226‡
Female (n=6)	Ovariectomy + Estradiol	2036±598‡

Each value is the mean \pm standard error of the mean.

^a Rats were neonatally sham-operated (sham) or ovariectomized and the estrogen treated rats were administered daily estradiol injections from 8 to 10 weeks of age. All rats were sacrificed at 10 weeks of age.

^{\ddagger} Mean value of the estradiol-treated group is statistically different (p<0.05) from that of the appropriate group not administered estradiol.

3.3.a. EFFECT OF TAMOXIFEN ON TOTAL HEPATIC CYTOCHROME P450 CONTENT

Total cytochrome P450 concentrations and protein concentrations were measured in hepatic microsomes prepared from individual tamoxifen-treated and vehicle-treated rats. Mean values of both concentrations and of the specific content of cytochrome P450 are shown in Table 3.3.a. Values of the protein and cytochrome P450 concentrations are dependent on the dilution of the final 105,000 x g pellet with sucrose solution but the ratio of these concentrations, expressed as the specific content of cytochrome P450, is independent of dilution and can be used to determine the effect of a treatment on total cytochrome P450 levels. The mean specific content of cytochrome P450 did not vary significantly (p>0.05) between treated groups and their respective controls indicating that treatment with tamoxifen had no effect on the total cytochrome P450 content in either male or female rats.

3.3.b. EFFECT OF NEONATAL OVARIECTOMY WITH AND WITHOUT ADMINISTRATION OF ESTRADIOL DURING ADULTHOOD ON TOTAL HEPATIC CYTOCHROME P450 CONTENT

Total cytochrome P450 concentration, protein concentration and specific content were also measured in hepatic microsomes prepared from individual neonatally ovariectomized and sham-operated rats and the mean values are presented in Table 3.3.b. Like tamoxifentreatment, neonatal ovariectomy and estradiol-treatment did not affect the specific content of cytochrome P450 in liver. Mean specific content of the neonatally ovariectomized and shamoperated rats was slightly less than that of the vehicle-treated rats in the tamoxifen study.

		1		
			Cytochrome	Specific
		Protein	P450	Content
Sex	Treatment ^a	Concentration (mg/mL)	Concentration (mg/mL)	(nmol P450/mg protein)
				· ·
Male (n=8)	Vehicle	24.01±0.96	30.42±1.74	1.28 ± 0.08
Male (n=9)	Tamoxifen	22.69±0.95	28.82±1.91	1.26 ± 0.06
		· · · ·	· · ·	
Female (n=8)	Vehicle	15.73±1.54	13.96±1.07	0.91±0.05
	T	20.2(+1.17	17 11 1 57	0.0410.04
remaie (n=9)	ramoxiten	20.26±1.17	1/.11±1.3/*	0.84±0.04

Table 3.3.a. Effect of tamoxifen treatment on hepatic microsomal protein concentration, cytochrome P450 concentration and specific content in adult male and female rats.

Each value is the mean \pm standard error of the mean.

^a Rats were treated at 8 weeks of age and sacrificed at 13 weeks of age.

Table 3.3.b. Effect of neonatal ovariectomy with and without administration of estradiol during adulthood on hepatic microsomal protein concentration, cytochrome P450 concentration and specific content in adult female rats.

Sex	Treatment ^a	Protein Concentration (mg/mL)	Cytochrome P450 Concentration (mg/mL)	Specific Content (nmol P450/mg protein)
Female (n=6)	Sham	21.27±1.98	16.27±1.80	0.77±0.20
Female (n=9)	Ovariectomy	25.24±1.40	16.61±1.24	0.66±0.09
Female (n=6)	Sham + Estradiol	24.87±3.43	15.42±1.80	0.65±0.06
Female (n=6)	Ovariectomy + Estradiol	27.48±1.63	15.17±1.06	0.56±0.06

Each value is the mean \pm standard error of the mean.

^a Rats were neonatally sham-operated (sham) or ovariectomized and the estrogen treated rats were administered daily estradiol injections from 8 to 10 weeks of age. All rats were sacrificed at 10 weeks of age.

3.4.a. EFFECT OF TAMOXIFEN ON TESTOSTERONE HYDROXYLATION

Cytochromes P450 are involved in the metabolism of various steroid hormones, such as testosterone. Due to the regio- and stereospecificity of the hydroxylation of the steroid nucleus, one of the most convenient and sensitive methods of obtaining information about the enzymatic function of many isozymes is to measure the hydroxylation of these steroids. Several research groups (Wood et al., 1983; Waxman et al., 1983; Ryan et al., 1984; Waxman et al., 1985; Sonderfan et al., 1987) have demonstrated that specific cytochrome P450 isozymes purified from rat liver microsomes catalyze distinct testosterone oxidation pathways. Using antibody-inhibition studies, Waxman et al. (1985) showed that in rat liver microsomes CYP3A is responsible for greater than 85% of testosterone 6β -hydroxylase activity. It has also been established that the levels of CYP2A1, CYP2B1, and CYP2C11 are reflected by testosterone 7 α -, 16 β -, and 2 α -hydroxylase activities, respectively (Wood *et al.*, 1983; Ryan et al., 1984; Waxman et al., 1983; Waxman et al., 1985). When combined with high performance liquid chromatography (HPLC), which is a favourable method for separating and characterizing low concentrations of steroid metabolites, this assay is very useful as many catalytic activities can be monitored simultaneously.

The testosterone hydroxylase assay was used in the present study to identify differences between the treated groups and their controls with respect to their specific catalytic activities. The testosterone metabolites were identified and quantified by HPLC analysis. Initially, authentic standards of 2α -, 6β -, 7α -, 11β -, 16α -, and 16β -hydroxytestosterone were resolved (Figure 3.1.) for identification of the metabolites. The order of elution and retention times of the reaction products were compared with those of the

standards to verify the identity of the compounds. Chromatograms representative of the metabolites separated by HPLC in microsomes prepared from vehicle-treated adult male and female rats are shown in Figures 3.2.a. and 3.2.b., respectively. A standard curve was determined for each metabolite every time the assay was performed and a typical standard curve representative of all of the standard curves obtained is displayed in Figure 3.3. Each microsomal sample was assayed in duplicate on three separate occasions and a mean value was calculated for each treatment group. The daily variability of results obtained with the assay was minimal (less than 5%).



Figure 3.1. Chromatogram of 2α -, 6β -, 7α -, 16α - and 16β -hydroxytestosterone standards separated by HPLC. A mixture of 2.0 nmol of each standard was incubated for 5 min at 37° C in a 1-mL incubation mixture as described in the Experimental. The mixture was spiked with 2.5 nmol of 11 β hydroxytestosterone as an internal standard. The metabolites were resolved at 40°C on a reversephase C₁₈ column and detected at 254 nm. The abbreviations designate the regio- and stereochemistry of the hydroxylated testosterone standards (e.g. 2α denotes 2α -hydroxytestosterone).



Figure 3.2. Representative HPLC chromatograms of testosterone metabolites produced in hepatic microsomes prepared from vehicle-treated adult male (a.) and vehicle-treated adult female (b.) rat livers. Testosterone (0.25 mM) was incubated for 5 min at 37°C in 1-mL incubation mixtures containing microsomes (0.3 nmol cytochrome P450) as described in the Experimental. At the termination of the reaction the mixture was spiked with 2.5 nmol of 11 β -hydroxytestosterone as an internal standard. The metabolites were resolved at 40°C on a reverse-phase C₁₈ column and detected at 254 nm. The abbreviations designate the regio- and stereochemistry of the hydroxylated testosterone standards (e.g. 2 α denotes 2 α -hydroxytestosterone).



Figure 3.3. Standard calibration curve for 2α -, 6β -, 7α -, 16α - and 16β -hydroxytestosterone. Standard testosterone metabolites (0.5, 1.0, 2.0 and 5.0 nmol/tube) were incubated for 5 min at 37° C in 1-mL incubation mixtures as described in the Experimental. Each sample was spiked with 2.5 nmol of internal standard (11 β -hydroxytestosterone). A 10 µL aliquot was analyzed by HPLC. The individual standards were resolved at 40°C on a reverse-phase C₁₈ column and detected at 254 nm. The ratio of the peak area of the standard to the peak area of the internal standard was plotted against the concentration of the standard. The symbols used represent: $-\bigcirc 2\alpha$ -, $-\square$ - 6β -, $-\bigtriangleup$ - 7α -, $-\nabla$ - 16α - and $-\diamondsuit$ - 16β -hydroxytestosterone.

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The testosterone hydroxylase activities of hepatic microsomes prepared from the tamoxifen-treated male and female rats are presented in Table 3.4.a. Testosterone 2α -hydroxylase activity was present in microsomes prepared from both the vehicle- and tamoxifen-treated male rats with no significant difference (p>0.05) between them. This activity was not detected in microsomes prepared from the tamoxifen-treated or vehicle-treated female rats.

Hepatic microsomes prepared from both tamoxifen- and vehicle-treated male and female rats hydroxylated testosterone at the 6β -position. The testosterone 6β -hydroxylase activity of microsomes prepared from the tamoxifen-treated males did not differ significantly from those of the vehicle-treated group. In contrast, microsomes prepared from the tamoxifen-treated female rats showed a statistically significant increase of 61% in the rate of 6β -hydroxytestosterone produced relative to that of the vehicle-treated female rats.

Testosterone 7α -hydroxylase activity was present in microsomes prepared from the tamoxifen-treated and vehicle-treated adult male and female rats. The level of testosterone 7α -hydroxylase activity of microsomes prepared from the male tamoxifen-treated rats was not significantly different from that of microsomes prepared from the vehicle-treated males. However, microsomes prepared from the tamoxifen-treated female rats had only 57% of the testosterone 7α -hydroxylase activity observed in the microsomes prepared from the vehicle-treated rats was not significantly. The testosterone 7α -hydroxylase activity observed in the microsomes prepared from the vehicle-treated female rats. This difference is statistically significant using the Student *t*-test (p<0.05).

Testosterone 16α -hydroxylase activity was observed in microsomes prepared from both tamoxifen-treated and vehicle-treated male rats with no significant difference between

Table 3.4.a.Effeadult male and fee	ect of tamoxifen treatme nale rats.	nt on hepatic micro	osomal testosterone	2α-, 6β-, 7α-, 16α-	and 16β-hydroxylase	activities in
			Testo (nmol hydroxy	sterone Metabolites testosterone/mg pro	tein/min)	
Sex	Treatment ^a	2α	6В	Τα	16α	16β
Male (n=8)	Vehicle	2.63±0.17	1.10±0.15	0.14±0.02	2.47±0.15	*
Male (n=9)	Tamoxifen	2.15±0.23	1.15 ± 0.13	0.23±0.03	2.08±0.22	*
Female (n=8)	Vehicle	ł	0.19±0.01	1.07±0.09	*	*
Female (n=9)	Tamoxifen	I	0.31±0.02†	0.62±0.06†	*	*
Each value is the ^a Rats were treatt indicates not d	mean ± standard error o ed at 8 weeks of age and etected.	f the mean. The inc sacrificed at 13 we	lividual samples we eks of age.	rre assayed in duplic	ate on three separate oc	casions.

* Values were below the limit of detection (< 0.10 ± 0.05 nmol hydroxytestosterone formed/mg protein/min).
⁺ Mean value of the tamoxifen-treated group is statistically different (p<0.05) from that of the vehicle-treated group.

them. Small amounts of 16α -hydroxytestosterone were present in the hepatic microsomes prepared from tamoxifen-treated and vehicle-treated female rats as observed on the chromatogram, but this activity could not be quantitated because the area under the peaks and the peak heights were below the limit of detection of the peak integrator (less than 0.10 ± 0.05 nmol hydroxytestosterone formed/mg protein/min).

Testosterone 16β -hydroxylase activity was also below the limit of quantitation for microsomes prepared from both male and female tamoxifen-treated and vehicle-treated rats.

Due to the lack of effect of tamoxifen on the testosterone hydroxylase acivities in the male rats, only microsomes prepared from female rats were studied further.

3.4.b. EFFECT OF NEONATAL OVARIECTOMY WITH AND WITHOUT ADMINISTRATION OF ESTRADIOL DURING ADULTHOOD ON TESTOSTERONE HYDROXYLATION

Table 3.4.b. displays the measured testosterone hydroxylase activities for hepatic microsomes prepared from neonatally ovariectomized and sham-operated female rats. As expected, testosterone 2α -hydroxylase activity was not detected in hepatic microsomes prepared from any of these groups. Unlike tamoxifen treatment, neither ovariectomy nor estradiol-treatment significantly affected hepatic microsomal 6β - or 7α -testosterone hydroxylase activity. The level of hepatic microsomal 6β -hydroxylase activity observed in neonatally ovariectomized and sham-operated rats was similar to that observed in vehicle-treated female rats from the tamoxifen study. The rate of testosterone 7α -hydroxylation in microsomes prepared from neonatally ovariectomized and sham-operated rats was less than

Table 3.4.b. Eff testosterone $2\alpha_{-}$,	cet of neonatal ovariecton 6β-, 7α-, 16α- and 16β-hy	ay with and with droxylase activiti	out administration es in adult female r	of estradiol during ad ats.	ulthood on hepatic n	nicrosomal
			Testo (nmol hydroxy	sterone Metabolites testosterone/mg protein	/min)	
Sex	Treatment ^a	2α	6β	7α	16α	16ß
Female (n=6)	Sham		0.21±0.04	0.65±0.17	*	*
Female (n=9)	Ovariectomy	I	0.19 ± 0.02	0.48±0.03	*	*
Female (n=6)	Sham + Estradiol	ł	0.16±0.02	0.71±0.13	*	×
Female (n=6)	Ovariectomy + Estradiol		0.17±0.02	0.56±0.05	*	*
Each value is the ^a Rats were nev injections from 8 indicates not d * Values were be	mean ± standard error of th matally sham-operated (sh to 10 weeks of age. All ra etected.	at mean. The ind nam) or ovariecto ts were sacrificed (< 0.10 ± 0.05 nm	ividual samples we omized and the es at 10 weeks of age ol hydroxytestoste	re assayed in duplicate tradiol treated rats we 	on three separate ocore administered dail.	asions. y estradiol

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in the microsomes prepared from vehicle-treated female rats in the tamoxifen study. As with the tamoxifen- and vehicle-treated female rats, chromatographic peaks corresponding to 16α and 16β -hydroxytestosterone in hepatic microsomes prepared from the neonatally ovariectomized and sham-operated rats were below the limit of quantitation.

3.5.a. EFFECT OF TAMOXIFEN ON p-NITROPHENOL HYDROXYLATION

The hydroxylation of *p*-nitrophenol has been shown to be a good indicator of CYP2E1 levels in the rat, rabbit and humans (Reinke and Moyer, 1985; Koop, 1986; Tassaneeyakul *et al.*, 1993). *p*-Nitrophenol was preferred over other CYP2E1 substrate probes because both aniline and paracetamol are oxidized by several other cytochrome P450 isoforms, and N-nitrosodimethylamine is a known carcinogen and radiolabelled compound is required to measure N-demethylase activity using this substrate (Tassaneeyakul *et al.*, 1993).

In earlier experiments conducted in our laboratory using the same assay conditions as those used in the present study, it was determined that the rate of product formation was linear with incubation time and microsomal protein concentration, and independent of substrate and NADPH concentrations. Quantitation was achieved using a standard calibration curve which displayed good linearity ($r^2=1.0$).

The *p*-nitrophenol hydroxylase activity was measured in duplicate for all of the samples and the mean values are displayed in Table 3.5.a. There was no significant difference (p>0.05) in *p*-nitrophenol hydroxylase activity between hepatic microsomes prepared from tamoxifen-treated female rats and vehicle-treated female rats. The mean value of this activity for microsomes prepared from the vehicle-treated rats agrees with the activity

reported by Reinke and Moyer (1985) for adult female rats (0.74±0.07 nmol 4-nitrocatechol

formed/min/mg protein).

Table 3.5.a. Effect of tamoxifen treatment on hepatic microsomal *p*-nitrophenol hydroxylase activity in adult female rats.

Treatment ^a	<i>p</i> -Nitrophenol Hydroxylase Activity (nmol 4-nitrocatechol formed/min/mg protein)
Vehicle (n=8)	0.75±0.11
Tamoxifen (n=9)	1.00±0.04

Each value is the mean \pm standard error of the mean.

^a Rats were treated at 8 weeks of age and sacrificed at 13 weeks of age.

3.5.b. EFFECT OF NEONATAL OVARIECTOMY WITH AND WITHOUT ADMINISTRATION OF ESTRADIOL DURING ADULTHOOD ON p-NITROPHENOL HYDROXYLATION

The *p*-nitrophenol hydroxylase activities of hepatic microsomes prepared from neonatally ovariectomized and sham-operated rats were also measured and the mean values for each group are presented in Table 3.5.b. Like tamoxifen treatment, neither neonatal ovariectomy nor estradiol-treatment significantly (p>0.05) affected hepatic microsomal *p*-nitrophenol hydroxylase activity. The *p*-nitrophenol hydroxylase activity of microsomes prepared from the neonatally ovariectomized and sham-operated rats was greater than that of microsomes prepared from the vehicle-treated female rats in the tamoxifen study.

Table	3.5.b.	Effect	of neor	natal o	ovariec	tomy	with	and	without	adm	inistrat	tion	of e	stradiol
during	adultho	ood on	hepatic	micro	osomal	p-niti	rophe	nol h	nydroxyla	ase a	ctivity	in	adult	female
rats.														

Treatment ^a	<i>p</i> -Nitrophenol Hydroxylase Activity (nmol 4-nitrocatechol formed/min/mg protein)
Sham (n=6)	1.38±0.10
Ovariectomy (n=9)	1.45±0.10
Sham + Estradiol (n=6)	1.02±0.11
Ovariectomy + Estradiol (n=6)	1.19±0.08

Each value is the mean \pm standard error of the mean.

^a Rats were neonatally sham-operated (sham) or ovariectomized and the estradiol treated rats were administered daily estradiol injections from 8 to 10 weeks of age. All rats were sacrificed at 10 weeks of age.

3.6. EFFECT OF TAMOXIFEN ON PENTOXYRESORUFIN O-DEPENTYLATION

Cytochromes P450 catalyze the depentylation of pentoxyresorufin to resorufin. With microsomes prepared from untreated rats PROD activity is very low and the activity is probably catalyzed by a number of cytochrome P450 isozymes. Using antibody-inhibition studies, Burke *et al.* (1994) demonstrated the extent to which each isozyme is involved (CYP1A1 < 20%, CYP1A2 < 20%, CYP2B1 < 20%, CYP2C6 \approx 20-40%, CYP2C11 \approx 20-40%, CYP3A1/2 \approx 20-40%). However, with microsomes prepared from phenobarbital-treated rats, Nakajima *et al.* (1990) reported that monoclonal antibodies against CYP2B inhibited PROD activity by at least 96%.

PROD activity was monitored in microsomes prepared from the tamoxifen-treated female rats because White *et al.* (1992) previously demonstrated a 60-fold increase in this

activity in microsomes prepared from adult female rats treated with large doses of tamoxifen (45 mg/kg *i.p.*) for four days and killed 24 h later. These investigators most likely saw a direct inductive effect of tamoxifen itself on cytochromes P450 but as we are interested in the influence of tamoxifen via its long term hormonal effects, we wanted to determine if any residual direct effects were still present.

The PROD activity of individual hepatic microsomes prepared from tamoxifentreated and vehicle-treated rats were determined in duplicate and the mean values are presented in Table 3.6. Quantitation was achieved using a standard calibration curve that displayed good linearity ($r^2=1.0$). The data shown in Table 3.6 indicates that there was very little PROD activity in microsomes from either the tamoxifen-treated or the vehicle-treated rats and the activity was not significantly affected by treatment with tamoxifen. The PROD assay was not performed on the other groups since little activity was expected as the isozymes involved in PROD activity in uninduced animals have not been shown to be affected by ovariectomy or estradiol levels.

Table 3.6. Effect of tamoxifen treatment on hepatic microsomal pentoxyresorufin O-depentylase (PROD) activity in adult female rats.

Treatment ^a	PROD Activity (nmol resorufin formed/min/mg protein)
Vehicle (n=8)	0.004±0.002*
Tamoxifen (n=9)	0.001±0.001*

Each value is the mean \pm standard error of the mean.

^a Rats were treated at 8 weeks of age and sacrificed at 13 weeks of age.

* Values are at or very near to baseline.

3.7. EFFECT OF TAMOXIFEN ON LEVELS OF INDIVIDUAL CYTOCHROMES P450

The enzyme activity data indicates that treatment with tamoxifen affected CYP2A1 and CYP3A-catalyzed testosterone hydroxylation but did not affect catalytic activities mediated by CYP2B or CYP2E1 in adult female rat hepatic microsomes. In order to determine if tamoxifen was exerting its effect at the level of protein expression, levels of the individual cytochrome P450 proteins were measured.

3.7.1. Initial attempt at immunoquantitation by competitive enzyme-linked immunosorbant assay (ELISA)

Competitive ELISA is a very sensitive immunoquantitation method that can be used to accurately measure the amount of specific antigens. Because of its sensitivity it was initially chosen to measure the expression of individual cytochromes P450 in the present study. As there is no initial separation of proteins the accuracy of this method is determined by the specificity of the antibody used, but all of the antibodies used in this study are monospecific.

Competitive ELISA was attempted to quantitate the protein level of CYP2A1, CYP2C7 and CYP3A. Initially, the assay conditions had to be optimized to ensure that the reaction was linear with respect to the concentration of the primary antibody, the concentration of the secondary antibody and time. Several experiments were conducted but the results were not consistent between assays and did not always display linear regions. After about two months of trying to set up a reliable assay and after consuming many microlitres of purified antibodies and proteins, it was decided to switch to immunoblots and densitometry to quantitate the cytochrome P450 protein levels. Densitometric analysis of immunoblots is not as sensitive a quantitative method as competitive ELISA but is an established and widely-used technique to measure expression of hepatic cytochromes P450 and if carefully employed should accurately reflect absolute as well as relative levels of individual cytochromes P450.

3.7.2. Quantitation by densitometric determination of protein levels

Monospecific, polyclonal antibodies raised against individual purified CYP2A1, CYP2C7, CYP2E1 and CYP3A, and monoclonal antibodies raised against purified CYP3A1 and CYP3A2 were used to probe nitrocellulose blots of microsomal proteins separated on SDS-polyacrylamide gels. The relative band intensities on the immunoblots were quantitated using a VISAGE 110 Bio Image Analyzer. Values were reported as band integrated intensity which is the optical density of the band centre (with the background subtracted) multiplied by the area. Where possible, cytochrome P450 isozyme contents were calculated with the use of a calibration curve in which staining intensities of known amounts of purified cytochrome P450 standards were plotted against the amount of purified cytochrome P450 protein loaded per lane. Otherwise, relative levels of individual immunoreactive cytochrome P450 proteins present in microsomes were determined on the basis of integrated band intensities only.

For the immunoblots, alkaline phosphatase (AP)-conjugated secondary antibodies were used instead of HRP-conjugated secondary antibodies because Waxman *et al.* (1991) reported AP-conjugated secondary antibodies as being superior in terms of colour quality, intensity, and long-term stability of the final blot to fading. For quantitative analysis of immunoblots, the assay conditions had to be optimized to ensure that colour development did not proceed beyond the linear response range of the phosphatase reaction. Therefore, the range of linearity of signal intensity was established with respect to protein concentration and time of reaction. A pooled sample of microsomes prepared from control female rats was loaded onto a gel at concentrations of 1.0, 0.75, 0.5, 0.25 and 0.125 mg/mL, subjected to electrophoresis as described in Experimental 2.9.2, blotted onto a nitrocellulose membrane and probed with anti-CYP2E1 polyclonal IgG. After incubation with AP-conjugated secondary antibody the blot was developed with the alkaline phosphatase substrate solution and the reaction was allowed to proceed for 1, 2.5, 5, 7.5, 10 or 15 minutes. The integrated intensities of the resulting immunostained bands were plotted against reaction time for each microsomal protein concentration as shown in Figure 3.4. Using an initial microsomal protein concentration of 0.5 mg/mL, which translates into 10 μ g of microsomal protein loaded onto the gel, the reaction was linear for up to 7.5 minutes.



Figure 3.4. A plot of integrated intensity versus time at varying protein concentrations for the alkaline phosphatase reaction. Adult female control rat microsomes were subjected to electrophoresis on a discontinuous polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The blot was immunostained using rabbit anti-CYP2E1 polyclonal IgG at a concentration of 10 µg IgG/mL. Secondary antibody (goat anti-rabbit IgG) was used at a dilution of 1:3000. The alkaline phosphatase reaction was terminated after 1, 2.5, 5, 7.5, 10 or 15 min. The intensity of the bands was measured using a VISAGE 110 Bio Image Analyzer. These methods are described in detail in the Experimental section. The symbols used represent: $-\bigcirc$ 2.5, $-\bigcirc$ 5.0, $-\bigtriangleup$ 10.0, $-\nabla$ -15.0 and $-\diamondsuit$ - 20.0 pmol of microsomal protein per lane.

3.7.2.1. Immunoquantitation of adult female rat hepatic microsomes probed with anti-CYP2A1 IgG

Figures 3.5.a., 3.5.b. and 3.5.c. show immunoblots of hepatic microsomes prepared from adult female rats probed with sheep anti-CYP2A1 polyclonal IgG. The antibody recognized a single band in all of the microsomal samples at the same position as purified rat CYP2A1 suggesting that the antibody was monospecific. The staining intensity of the immunoreactive band in each of the lanes containing microsomes from the tamoxifen-treated rats (Figure 3.5.a.) appeared to be fainter than that from the vehicle-treated rats suggesting that CYP2A1 was present in a lesser amount in microsomes from rats treated with tamoxifen. Likewise, the staining intensity of the immunoreactive band in each of the lanes containing microsomes from the neonatally ovariectomized rats (Figure 3.5.b.) appeared to be fainter than that from the neonatally sham-operated rats suggesting that CYP2A1 was present in a lesser amount in microsomes from the neonatally ovariectomized rats. The staining intensity of the immunoreactive band in the lanes containing microsomes from the neonatally ovariectomized rats administered estradiol during adulthood (Figure 3.5.c.) appeared to be the same as that from the neonatally sham-operated rats administered estradiol during adulthood suggesting that CYP2A1 is present in the same amount in both of these groups. The blots were scanned to quantitate band intensities. A known concentration of purified CYP2A1 was loaded onto each of the gels and the blots were normalized with respect to staining intensity. Mean values of the densitometry data are given in Tables 3.7.a. and 3.7.b.

Table 3.7.a. demonstrates the effects of tamoxifen treatment on hepatic levels of CYP2A1 expressed in terms of amounts of immunoreactive protein, CYP2A1 content (pmol/mg protein) and CYP2A1 as a percent of total cytochrome P450. The amount of

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Figure 3.5. Immunoblots of adult female rat hepatic microsomes probed with polyclonal antibody against rat CYP2A1. All microsomal samples were applied to the gel at a final concentration of 10 μg of microsomal protein per lane. Primary antibody (sheep anti-CYP2A1 polyclonal IgG) was used at a concentration of 40 μg IgG/mL, secondary antibody (rabbit anti-sheep IgG) was used at a dilution of 1:250, and the alkaline phosphatase reaction was terminated after 7 min. **a.** Lanes 1 to 8 contain microsomes from vehicle-treated adult female rats, lane 9 contains 0.250 pmol purified cytochrome 2A1, and lanes 10 to 18 contain microsomes from tamoxifen-treated adult female rats. **b.** Lanes 2 to 7 contain microsomes from neonatally sham-operated adult female rats, lane 9 contains 0.125 pmol purified CYP2A1, and lanes 1, 8, 10 to 14, 17 and 18 contain microsomes from neonatally ovariectomized adult female rats. (Lanes 15 and 16 were contaminated with other microsomes.) **c.** Lanes 1 to 6 contain microsomes from neonatally sham-operated adult female rats administered estradiol during adulthood, lanes 7 to 12 contain 0.03125, 0.0625, 0.125, 0.250, 0.375 and 0.500 pmol purified CYP2A1, respectively, and lanes 13 to 18 contain microsomes from neonatally ovariectomized adult female rats administered estradiol during adulthood, lanes 7 to 12 contain 0.03125, 0.0625, 0.125, 0.250, 0.375 and 0.500 pmol purified CYP2A1, respectively, and lanes 13 to 18 contain microsomes from neonatally ovariectomized adult female rats administered estradiol during adulthood.

Treatment ^a	Amount of Immunoreactive Protein ^b	Cytochrome P450 2A1 Content (pmol/mg protein)	Cytochrome P450 2A1 as a Percent of Total Cytochrome P450
Vehicle (n=8)	52.30±4.11	40.24±3.16	4.42±0.35
Tamoxifen (n=9)	36.96±0.87†	28.45±0.67†	3.39±0.08†

Table 3.7.a. Effect of tamoxifen treatment on hepatic microsomal cytochrome P450 2A1 levels in adult female rats.

Each value is the mean \pm standard error of the mean.

^a Rats were treated at 8 weeks of age and sacrificed at 13 weeks of age.

^b Values were determined by densitometric analysis of an immunoblot probed with monospecific antibody against rat CYP2A1 and expressed as the integral of optical density of the band per milligram protein.

[†] Mean value of the tamoxifen-treated group is statistically different (p<0.05) from that of the vehicle-treated group.

immunoreactive protein is a relative term and is expressed as the integral of optical density of the immunostained band per milligram of microsomal protein loaded onto the gel. To calculate the CYP2A1 content, a calibration curve (Figure 3.6.) was constructed from the values of integrated intensity of different known amounts of purified rat CYP2A1 loaded onto the gel (Figure 3.4.c.). Knowing the specific content of the microsomes (nmol P450/mg protein), CYP2A1 as a percent of total cytochrome P450 was easily expressed.

Hepatic microsomal CYP2A1 levels in the tamoxifen-treated rats were about 71% of the levels in the vehicle-treated rats which is a statistically significant difference (p<0.05) and suggests that tamoxifen treatment suppresses the expression of CYP2A1. CYP2A1 levels in microsomes prepared from neonatally ovariectomized and sham-operated rats are displayed

Treatment ^a	Amount of Immunoreactive Protein ^b	Cytochrome P450 2A1 Content (pmol/mg protein)	Cytochrome P450 2A1 as a Percent of Total Cytochrome P450
Sham (n=6)	24.21±1.11	18.64±0.86	2.42±0.11
Ovariectomy (n=9)	20.03±0.37 ⁺	15.42±0.28 ⁺	2.34±0.04
Sham + Estradiol (n=6)	33.20±1.66‡	25.55±1.27‡	3.92±0.20‡
Ovariectomy + Estradiol (n=6)	32.28±0.90‡	24.85±0.69‡	4.44±0.12‡

Table 3.7.b. Effect of neonatal ovariectomy with and without administration of estradiol during adulthood on hepatic microsomal cytochrome P450 2A1 levels in adult female rats.

Each value is the mean \pm standard error of the mean.

^a Rats were neonatally sham-operated (sham) or ovariectomized and the estradiol treated rats were administered daily estradiol injections from 8 to 10 weeks of age. All rats were sacrificed at 10 weeks of age.

^b Values were determined by densitometric analysis of an immunoblot probed with monospecific antibody against rat CYP2A1 and expressed as the integral of optical density of the band per milligram protein.

[†] Mean value of the ovariectomized group is statistically different (p<0.05) from that of the shamoperated group.

^{\ddagger} Mean value of the estradiol-treated group is statistically different (p<0.05) from that of the appropriate group not administered estradiol.

in Table 3.7.b. As with tamoxifen treatment, the mean CYP2A1 level in microsomes prepared from neonatally ovariectomized rats was significantly lower (about 83%) than that from the neonatally sham-operated rats, suggesting that ovariectomy suppresses CYP2A1 expression. In contrast, the mean CYP2A1 level of microsomes prepared from the neonatally ovariectomized rats administered estradiol during adulthood was not significantly different from that of the neonatally sham-operated rats administered estradiol during adulthood.

However, the hepatic microsomal CYP2A1 levels in the estradiol-treated groups were significantly higher than those in the appropriate groups not administered estradiol, suggesting that estradiol has a positive influence on the expression of CYP2A1.



Figure 3.6. A plot of integrated intensity versus amount of purified CYP2A1 (pmol)/lane. Pure CYP2A1 (0.0625, 0.125, 0.250, 0.375 and 0.500 pmol/lane) was subjected to electrophoresis on a discontinuous polyacrylamide gel containing a 7.5% polyacrylamide separating gel that was 0.75 mm thick and 12.5 cm long and electrophoretically transferred to a nitrocellulose membrane. The blot was immunostained using a primary antibody (sheep anti-CYP2A1 polyclonal IgG) concentration of 40 μ g IgG/mL, a secondary antibody (rabbit anti-sheep IgG) at a 1:250 dilution, and the alkaline phosphatase reaction was terminated after 7 min. The intensity of the bands was measured using a VISAGE 110 Bio Image Analyzer. These methods are described in detail in the Experimental section. Concentrations of purified CYP2A1 less than 0.250 pmol/lane were below the limit of detection at the antibody concentrations used.

3.7.2.2. Immunoquantitation of adult female rat hepatic microsomes probed with anti-CYP2C7 IgG

Figures 3.7.a., 3.7.b. and 3.7.c. display immunoblots of hepatic microsomes prepared from adult female rats probed with rabbit anti-CYP2C7 polyclonal IgG. The antibody recognized a single band in all of the samples at the same position as purified rat CYP2C7 suggesting that this antibody is monospecific. The staining intensity of the immunoreactive band in each of the lanes containing microsomes from the tamoxifen-treated rats (Figure 3.7.a.) appears about the same as that from the vehicle-treated rats suggesting that CYP2C7 was not affected by tamoxifen treatment. The staining intensity of the immunoreactive band in the lanes containing microsomes from the neonatally ovariectomized rats (Figure 3.7.b.) and the neonatally ovariectomized rats administered estradiol during adulthood (Figure 3.7.c.) appeared fainter than that from the neonatally sham-operated rats and the neonatally sham-operated rats administered estradiol during adulthood, respectively, suggesting that CYP2C7 was present in a lesser amount in microsomes from the neonatally ovariectomized rats. The blots were scanned to quantitate band intensities. Mean values of the densitometry data are given in Tables 3.8.a. and 3.8.b.

The effect of tamoxifen treatment on hepatic microsomal CYP2C7 protein levels is displayed in Table 3.8.a. Unfortunately, the band intensities of the various concentrations of purified CYP2C7 present were too faint to quantitate and a standard curve could not be constructed. As a consequence, the CYP2C7 levels were only measured on a relative basis against their controls. There was no significant difference in the CYP2C7 level of the hepatic microsomes from the tamoxifen-treated rats and those from the vehicle-treated rats, suggesting that tamoxifen treatment does not affect the expression of CYP2C7. Table



Figure 3.7. Immunoblots of adult female rat hepatic microsomes probed with polyclonal antibody against rat CYP2C7. All microsomal samples were applied to the gel at a final concentration of 10 μ g of microsomal protein per lane. Primary antibody (rabbit anti-CYP2C7 polyclonal IgG) was used at a concentration of 100 μ g IgG/mL, secondary antibody (goat anti-rabbit IgG) was used at a dilution of 1:1000, and the alkaline phosphatase reaction was terminated after 6.5 min. **a.** Lanes 1 to 8 contain microsomes from vehicle-treated adult female rats, lane 9 contains 0.250 pmol purified CYP2C7, and lanes 10 to 18 contain microsomes from tamoxifen-treated adult female rats. **b.** Lanes 1 to 6 contain microsomes from neonatally sham-operated adult female rats, lane 7 contains 0.125 pmol purified CYP2C7, and lanes 8 to 16 contain microsomes from neonatally ovariectomized adult female rats administered estradiol during adulthood, lanes 7 to 12 contain 0.03125, 0.0625, 0.125, 0.250, 0.375 and 0.500 pmol purified CYP2C7, respectively, and lanes 13 to 18 contain microsomes from neonatally ovariectomized adult female rats administered estradiol during adulthoed, lanes 7 to 12 contain 0.03125, 0.0625, 0.125, 0.250, 0.375 and 0.500 pmol purified CYP2C7, respectively, and lanes 13 to 18 contain microsomes from neonatally ovariectomized adult female rats administered estradiol during adulthoed, lanes 7 to 12 contain 0.03125, 0.0625, 0.125, 0.250, 0.375 and 0.500 pmol purified CYP2C7, respectively, and lanes 13 to 18 contain microsomes from neonatally ovariectomized adult female rats administered estradiol during adulthoed.

Table 3.8.a. Effect of tamoxifen treatment on hepatic microsomal cytochrome P450 2C7 levels in adult female rats.

Treatment ^a	Amount of Immunoreactive Protein ^b		
Vehicle (n=8)	17.1±4.2		
Tamoxifen (n=9)	14.4±4.1		

Each value is the mean \pm standard error of the mean.

^a Rats were treated at 8 weeks of age and sacrificed at 13 weeks of age.

^b Values were determined by densitometric analysis of an immunoblot probed with monospecific antibody against rat CYP2C7 and expressed as the integral of optical density of the band per milligram protein.

Treatment ^a	Amount of Immunoreactive Protein ^b		
Sham (n=6)	22.5±3.0		
Ovariectomy (n=9)	6.0±1.3 [†]		
Sham + Estradiol (n=6)	22.5±3.5		
Ovariectomy + Estradiol (n=6)	19.8±1.7‡		

Table 3.8.b. Effect of neonatal ovariectomy with and without administration of estradiol during adulthood on hepatic microsomal cytochrome P450 2C7 levels in adult female rats.

Each value is the mean \pm standard error of the mean.

^a Rats were neonatally sham-operated (sham) or ovariectomized and the estradiol treated rats were administered daily estradiol injections from 8 to 10 weeks of age. All rats were sacrificed at 10 weeks of age.

^b Values were determined by densitometric analysis of an immunoblot probed with monospecific antibody against rat CYP2C7 and expressed as the integral of optical density of the band per milligram protein.

^{\dagger} Mean value of the ovariectomized group is statistically different (p<0.05) from that of the shamoperated group.

[‡] Mean value of the estradiol-treated group is statistically different (p<0.05) from that of the appropriate group not administered estradiol.

3.8.b. shows the relative amounts of CYP2C7 present in microsomes prepared from neonatally ovariectomized and sham-operated rats. In contrast to tamoxifen treatment, the amount of CYP2C7 in microsomes prepared from the neonatally ovariectomized rats was only 27% of the amount found in microsomes prepared from the sham-operated rats, suggesting that neonatal ovariectomy has a negative influence on the expression of CYP2C7. CYP2C7 expression did not vary significantly between the microsomes prepared from the neonatally ovariectomized rats administered estradiol during adulthood and those from the neonatally sham-operated rats administered estradiol during adulthood. However, microsomes prepared from the neonatally ovariectomized rats administered rats administered rats administered estradiol during adulthood. However, microsomes prepared from the neonatally ovariectomized rats administered rats administered rats administered estradiol during adulthood had a significantly greater amount of CYP2C7 than microsomes prepared from the neonatally ovariectomized rats without the estradiol treatment.

3.7.2.3. Immunoquantitation of adult female rat hepatic microsomes probed with anti-CYP2E1 IgG

Figures 3.8.a., 3.8.b. and 3.8.c. display the immunoblots of hepatic microsomes prepared from adult female rats probed with rabbit anti-CYP2E1 polyclonal IgG. The antibody recognized a single band in all of the microsomal samples suggesting that the antibody is monospecific. A purified standard was not present on the blots because purified CYP2E1 was not available. The staining intensity of the immunoreactive band in each of the lanes containing microsomes from the tamoxifen-treated rats (Figure 3.8.a.) appeared to be the same as that from the vehicle-treated rats suggesting that CYP2E1 was not affected by tamoxifen treatment. The staining intensity of the immunoreactive band in each of the lanes containing microsomes from the neonatally ovariectomized rats (Figure 3.8.b.) and the



Figure 3.8. Immunoblots of adult female rat hepatic microsomes probed with polyclonal antibody against rat CYP2E1. All microsomal samples were applied to the gel at a final concentration of 10 μ g of microsomal protein per lane. Primary antibody (rabbit anti-CYP2E1 polyclonal IgG) was used at a concentration of 10 μ g IgG/mL, secondary antibody (goat anti-rabbit IgG) was used at a dilution of 1:3000, and the alkaline phosphatase reaction was terminated after 5 min. **a.** Lanes 1 to 8 contain microsomes from vehicle-treated adult female rats, lane 9 contains no microsomes, and lanes 10 to 18 contain microsomes from tamoxifen-treated adult female rats. **b.** Lanes 1 to 6 contain microsomes from neonatally sham-operated adult female rats, lanes 7 and 8 contain no microsomes, and lanes 9 to 17 contain microsomes from neonatally sham-operated adult female rats administered estradiol during adulthood and lanes 7 to 12 contain microsomes from neonatally ovariectomized adult female rats administered estradiol during adulthood.

neonatally ovariectomized rats administered estradiol during adulthood (Figure 3.8.c.) appeared to be the same as that from the neonatally sham-operated rats and the neonatally sham-operated rats administered estradiol during adulthood, respectively, suggesting that CYP2E1 levels are not affected by neonatal ovariectomy. The blots were scanned to quantitate band intensities. Mean values of the densitometry data are given in Tables 3.9.a. and 3.9.b.

Microsomal CYP2E1 levels for the tamoxifen- and vehicle-treated rats are presented in Table 3.9.a. CYP2E1 could only be measured in relative terms since no purified CYP2E1 was available. The amount of immunoreactive protein expressed in microsomes prepared from the tamoxifen-treated rats did not vary significantly from that of the microsomes prepared from the vehicle-treated rats, suggesting that tamoxifen treatment does not affect CYP2E1 levels. Table 3.9.b. displays relative amounts of CYP2E1 expressed in the neonatally ovariectomized rats. Like tamoxifen treatment, neonatal ovariectomy had no significant effect on hepatic microsomal CYP2E1 levels. However, the microsomes prepared from the neonatally ovariectomized rats administered estradiol during adulthood had significantly lower levels of CYP2E1 than the microsomes prepared from the neonatally ovariectomized rats that did not receive estradiol during adulthood, suggesting that estradiol has a negative influence on the expression of CYP2E1.

Treatment ^a	Amount of Immunoreactive Protein ^b
Vehicle (n=8)	23.0±3.1
Tamoxifen (n=9)	22.4±2.5

Table 3.9.a. Effect of tamoxifen treatment on hepatic microsomal cytochrome P450 2E1 levels in adult female rats.

Each value is the mean \pm standard error of the mean.

^a Rats were treated at 8 weeks of age and sacrificed at 13 weeks of age.

^b Values were determined by densitometric analysis of an immunoblot probed with monospecific antibody against rat CYP2E1 and expressed as the integral of optical density of the band per milligram protein.

Table 3.9.b.	Effect of neonatal	ovariectomy	with an	d without	administration	of estradiol
during adulthe	ood on hepatic micro	osomal cytoch	rome P4	50 2E1 le	vels in adult fer	nale rats.

Treatment ^a	Amount of Immunoreactive Protein ^b		
Sham (n=6)	13.5±1.7		
Ovariectomy (n=9)	17.0±1.3		
Sham + Estradiol (n=6)	8.3±1.5		
Ovariectomy + Estradiol (n=6)	9.0±1.4‡		

Each value is the mean \pm standard error of the mean.

^a Rats were neonatally sham-operated (sham) or ovariectomized and the estradiol treated rats were administered daily estradiol injections from 8 to 10 weeks of age. All rats were sacrificed at 10 weeks of age.

^b Values were determined by densitometric analysis of an immunoblot probed with monospecific antibody against rat CYP2E1 and expressed as the integral of optical density of the band per milligram protein.

^{\ddagger} Mean value of the estradiol-treated group is statistically different (p<0.05) from that of the appropriate group not administered estradiol.

3.7.2.4. Immunoquantitation of adult female rat hepatic microsomes probed with anti-CYP3A IgG

Figures 3.9.a., 3.9.b. and 3.9.c. display the immunoblots of hepatic microsomes prepared from adult female rats probed with rabbit anti-CYP3A polyclonal IgG (backabsorbed). The antibody recognized a single band in all of the samples at the same position as purified rat CYP3A1. The specificity of this antibody had previously been assessed in our laboratory using noncompetitive ELISA with various purified rat cytochrome P450 isozymes and using immunoblots with different rat liver microsomal preparations. The antibody is not completely monospecific but recognizes predominantly CYP3A1. It also reacts with CYP3A2, but to a much lesser extent. The staining intensity of the immunoreactive band in each of the lanes containing microsomes from the tamoxifen-treated rats (Figure 3.9.a.) appeared to be the same as that from the vehicle-treated rats suggesting that CYP3A was not affected by tamoxifen treatment. The staining intensity of the immunoreactive band in each of the lanes containing microsomes from the neonatally ovariectomized rats (Figure 3.9.b.) and the neonatally ovariectomized rats administered estradiol during adulthood (Figure 3.9.c.) appeared to be the same as that from the neonatally sham-operated rats and the neonatally sham-operated rats administered estradiol during adulthood, respectively, suggesting that CYP3A levels are not affected by neonatal ovariectomy. The blots were scanned to quantitate band intensities. Mean values of the densitometry data are given in Tables 3.10.a. and 3.10.b.

The levels of CYP3A in microsomes prepared from tamoxifen- and vehicle-treated rats are presented in Table 3.10.a. A calibration curve was constructed from the integrated intensity values resulting from known concentrations of purified CYP3A1 (Figure 3.9.c.) and is shown in Figure 3.10. Hepatic microsomal CYP3A levels were not significantly different between the tamoxifen-treated rats and the vehicle-treated rats, suggesting that tamoxifen treatment does not affect CYP3A expression. Table 3.10.b. displays the microsomal cytochrome P450 levels of the ovariectomized rats. Like tamoxifen treatment, neonatal


Figure 3.9. Immunoblots of adult female rat hepatic microsomes probed with polyclonal antibody against rat CYP3A. All microsomal samples were applied to the gel at a final concentration of 10 µg of microsomal protein per lane. Primary antibody (rabbit anti-CYP3A polyclonal IgG) was used at a concentration of 50 µg IgG/mL, secondary antibody (goat anti-rabbit IgG) was used at a dilution of 1:3000, and the alkaline phosphatase reaction was terminated after 7 min. **a.** Lanes 1 to 8 contain microsomes from vehicle-treated adult female rats, lane 9 contains 0.250 pmol purified CYP3A, and lanes 10 to 18 contain microsomes from tamoxifen-treated adult female rats. **b.** Lanes 1 to 6 contain microsomes from neonatally sham-operated adult female rats, lane 7 contains 0.250 pmol purified CYP3A, and lanes 1 to 6 contain microsomes from neonatally sham-operated adult female rats administered estradiol during adulthood, lanes 7 to 12 contain 0.0625, 0.125, 0.250, 0.375, 0.500 and 0.03125 pmol purified CYP3A, respectively, and lanes 13 to 18 contain microsomes from neonatally ovariectomized adult female rats administered estradiol during adulthood.

ovariectomy did not significantly affect hepatic microsomal CYP3A expression. However, microsomes prepared from the estradiol-treated groups had significantly lower levels of CYP3A than microsomes prepared from the appropriate groups not administered estradiol, suggesting that estradiol suppresses the expression of CYP3A.

In order to determine whether CYP3A1 or CYP3A2 was expressed in the female rats in the present study, blots were probed with monoclonal mouse anti-CYP3A1 IgG and monoclonal mouse anti-CYP3A2 IgG. Neither antibody recognized any proteins in hepatic microsomes prepared from tamoxifen-treated or vehicle-treated adult female rats (immunoblots not shown).

Treatment ^a	Amount of Immunoreactive Protein ^b	Cytochrome P450 3A Content (pmol/mg protein)	Cytochrome P450 3A as a Percent of Total Cytochrome P450
Vehicle (n=8)	1.88±0.28	7.45±0.93	0.82±0.10
Tamoxifen (n=9)	1.39±0.34	5.84±1.11	0.70±0.13

Table 3.10.a. Effect of tamoxifen treatment on hepatic microsomal cytochrome P450 3A levels in adult female rats.

Each value is the mean \pm standard error of the mean.

^a Rats were treated at 8 weeks of age and sacrificed at 13 weeks of age.

^b Values were determined by densitometric analysis of an immunoblot probed with monospecific antibody against rat CYP3A and expressed as the integral of optical density of the band per milligram protein.

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Treatment ^a	Amount of Immunoreactive Protein ^b	Cytochrome P450 3A Content (pmol/mg protein)	Cytochrome P450 3A as a Percent of Total Cytochrome P450
Sham (n=6)	1.69±0.37	6.82±1.20	0.89±0.16
Ovariectomy (n=9)	1.44±0.25	6.01±0.83	0.91±0.13
Sham + Estradiol (n=6)	1.18±0.08	1.18±0.09‡	0.18±0.01‡
Ovariectomy + Estradiol (n=6)	1.33±0.09	1.33±0.09‡	0.24±0.02‡

Table 3.10.b. Effect of neonatal ovariectomy with and without administration of estradiol during adulthood on hepatic microsomal cytochrome P450 3A levels in adult female rats.

Each value is the mean \pm standard error of the mean.

^a Rats were neonatally sham-operated (sham) or ovariectomized and the estradiol treated rats were administered daily estradiol injections from 8 to 10 weeks of age. All rats were sacrificed at 10 weeks of age.

^b Values were determined by densitometric analysis of an immunoblot probed with monospecific antibody against rat CYP3A and expressed as the integral of optical density of the band per milligram protein.

^{\ddagger} Mean value of the estradiol-treated group is statistically different (p<0.05) from that of the appropriate group not administered estradiol.



Figure 3.10. A plot of integrated intensity versus amount of purified CYP3A1 (pmol)/lane. Pure CYP3A1 (0.0625, 0.125, 0.250, 0.375 and 0.500 pmol/lane) was subjected to electrophoresis on a discontinuous polyacrylamide gel containing a 7.5% polyacrylamide separating gel that was 0.75 mm thick and 12.5 cm long and electrophoretically transferred to a nitrocellulose membrane. The blot was immunostained using a primary antibody (rabbit anti-CYP3A polyclonal IgG) concentration of 50 μ g IgG/mL, a secondary antibody (goat anti-rabbit IgG) at a 1:3000 dilution, and the alkaline phosphatase reaction was terminated after 7 min. The intensity of the bands was measured using a VISAGE 110 Bio Image Analyzer. These methods are described in detail in the Experimental section.

4. **DISCUSSION**

The present study examined the effects of tamoxifen, administered at a dose associated with antineoplastic activity and suppression of GH secretion in rats, on hepatic microsomal cytochrome P450-mediated catalytic activities and cytochrome P450 protein levels in adult female rats. The effects of tamoxifen treatment were compared to the effects of neonatal ovariectomy, with or without subsequent treatment with estradiol during adulthood, on cytochrome P450 expression with the aim of determining the influence of estradiol on these parameters. The purpose of the investigation was to test our hypothesis that tamoxifen would affect the expression of the cytochrome P450 isozymes that are hormonally regulated, either by suppression of GH secretion or by blocking the action of estrogen.

We chose to investigate several cytochrome P450 isozymes that are hormonally controlled, specifically CYP2A1, CYP2B1/CYP2B2, CYP2C7, CYP2C11, CYP2E1 and CYP3A. We considered including CYP2C12, a female-specific isozyme known to be regulated by GH and estradiol levels (MacGeoch *et al.*, 1984; Dannan *et al.*, 1986), in this study but ultimately did not because antibody against CYP2C12 was not available (from commercial sources or other research groups with whom we collaborate) and an enzyme assay to measure the 15 β -hydroxylation of 5 α -androstane-3 α ,17 β -diol 3,17-disulphate, its only major catalytic activity (MacGeoch *et al.*, 1984), is not feasible at present as we have not been able to locate a source for the substrate.

Although our expectation was that tamoxifen would alter the expression of hepatic cytochromes P450, there was a possibility that, five weeks after dosing, residual tamoxifen or its metabolites would competitively inhibit or activate one or more cytochrome P450 enzymes because tamoxifen was given at a relatively large dose and tamoxifen is known to be metabolized by several different cytochrome P450 isozymes in the rat. This possibility was explored in the following ways: (i) both the enzyme activities and protein levels were measured to determine whether the effects were a result of induction versus activation or suppression versus inhibition, (ii) enzyme activity was measured in male rats as well as female rats to determine if the effects were due to activation or inhibition (i.e. not sex-related) or if the effects were hormone-mediated (i.e. sex-related), and (iii) hepatic microsomal PROD activity was measured because White *et al.* (1992) reported a dramatic increase in this activity shortly after the administration of large doses of tamoxifen suggesting that tamoxifen itself has a direct inductive effect.

4.1. COMPARISON OF THE EFFECTS OF TAMOXIFEN AND NEONATAL OVARIECTOMY ON BODY WEIGHT AND LIVER WEIGHT

Body weight is dependent on many factors including age, sex, nutrition, genetic constitution, disease state and endocrine function. Both the male and female rats administered tamoxifen in this study had significantly lower body weights five weeks after treatment than the appropriate vehicle-treated controls (Table 3.1.a.). Tannenbaum *et al.* (1992) showed that rats treated with tamoxifen using the same dosing regimen as in the present study had dramatically reduced plasma GH levels. Although they did not report body weights for the rats in their study, GH is known to be an essential endocrine component for

growth. Earlier studies in which serum GH levels were reduced by hypophysectomy (Waxman *et al.*, 1991), neonatal administration of MSG (Millard *et al.*, 1982; Pampori *et al.*, 1991) or by a genetic mutation causing GH deficiency (Charlton *et al.*, 1988) reported a significant decrease in body weight gain. The decrease in body weight gain observed in the present study is most likely due to a decrease in GH secretion, which supports the assumption that the tamoxifen-treated rats have reduced GH levels as shown by Tannenbaum's study. Thus, although we did not measure serum GH levels, there is strong indirect evidence that GH levels were suppressed.

Unlike tamoxifen treatment, neonatal ovariectomy did not significantly affect final body weight (Table 3.1.b.). Previous studies have shown that neonatally ovariectomized adult female rats have significantly (p<0.01) greater body weights than neonatally shamoperated adult female rats (Bell and Zucker, 1971; Jansson *et al.*, 1985c). The lack of effect of ovariectomy on body weight in our study is most likely due to the absence of a change in serum estradiol levels in the ovariectomized rats (see 4.2.). Estradiol treatment resulted in slightly, yet not significantly, decreased body weight as compared to rats not administered estradiol (Table 3.1.b.). Estradiol has been reported as having a weight-limiting effect in adult gonadectomized rats of both sexes (Sullivan and Smith, 1957; Kakolewski *et al.*, 1968; Wade and Gray, 1979). The suppressive action of estradiol on body weight further indicates that tamoxifen decreases body weight gain via its suppression of GH and not as a result of blocking estrogen binding. Therefore, for the purpose of interpreting the results of the present study, I will assume that GH levels were suppressed in the tamoxifen-treated rats.

4.2. COMPARISON OF THE EFFECTS OF TAMOXIFEN AND NEONATAL OVARIECTOMY ON SERUM ESTRADIOL LEVELS

Rats have a four day estrous cycle and estradiol levels in normal adult female rats have been reported to range from 17 ± 2 pg/mL day one to 88 ± 2 pg/mL day four (Butcher *et al.*, 1974; Dohler and Wuttke, 1975; Belanger *et al.*, 1981). We did not determine at which day of the estrous cycle the rats in this study were but the mean estradiol level in the vehicletreated female rats in this study was almost twice that of the higher value reported above. The mean serum estradiol level in the tamoxifen-treated rats did not vary significantly from that of the vehicle-treated rats (Table 3.2.a.), suggesting that the tamoxifen treatment used does not affect serum estradiol levels. This result was not unexpected since the level of circulating estradiol remains low in postmenopausal women on tamoxifen therapy (Love, 1989).

Like tamoxifen treatment, neonatal ovariectomy did not cause a significant change in serum estradiol concentrations as compared to the sham-operated rats(Table 3.2.b.). Jansson and Frohman (1987b) previously reported a mean serum estradiol level of 14.8 ± 1.1 pg/mL for neonatally ovariectomized adult female rats which was significantly lower (p<0.05) than that of the neonatally sham-operated adult female rats (26.2±3.1 pg/mL). We have no explanation for the discrepancy in the effect of neonatal ovariectomy on serum estradiol levels between our study and that of Jansson and Frohman (1987b).

Estradiol administration during adulthood significantly increased the mean serum estradiol level about 20-fold relative to that of the appropriate groups not administered estradiol (Table 3.2.b.). Therefore, it is expected that the acute effects of estradiol on various physiological or biochemical endpoints would be manifested in these two groups of rats.

However, these effects may not always be obvious. For example, rats administered estradiol from eight to ten weeks of age did have substantially greater serum estradiol levels at the time of death than the rats not administered estradiol, but this estradiol treatment did not significantly decrease body weight. This effect might have been more pronounced if estradiol administration was started before the rats were adults. Alternately, two weeks might not have been a long enough period of time in which a significant effect on body weight could be observed.

4.3. COMPARISON OF THE EFFECTS OF TAMOXIFEN AND NEONATAL OVARIECTOMY ON TOTAL HEPATIC MICROSOMAL CYTOCHROME P450 CONTENT

Hepatic cytochrome P450 content is a crude measure of the xenobiotic oxidative capability of an animal. The total hepatic microsomal cytochrome P450 level in female rats is 10 to 30% lower than that of male rats (Waxman *et al.*, 1985). In our study, vehicle-treated female rats had a 29% lower total hepatic microsomal cytochrome P450 content than vehicle-treated male rats.

Tamoxifen treatment did not significantly affect the total hepatic microsomal cytochrome P450 content in male or female rats as compared to the vehicle-treated male and female rats, respectively (Table 3.3.a.). Similarly, neither neonatal ovariectomy nor estradiol treatment significantly affected the total hepatic microsomal cytochrome P450 content compared to the appropriate control groups (Table 3.3.b.).

4.4. EVIDENCE THAT THE EFFECTS OF TAMOXIFEN ON CYTOCHROME P450 EXPRESSION ARE THROUGH A SEX-RELATED, HORMONE-MEDIATED MECHANISM

Hydroxylation of testosterone at the 2α - and 16α -positions in hepatic microsomes from uninduced male rats is catalyzed predominantly by CYP2C11, a male-specific isozyme (Morgan *et al.*, 1985; Waxman, 1984). In this study, hepatic microsomal testosterone 2α and 16α -hydroxylase activities in adult male and female rats were not affected by tamoxifen treatment (Table 3.4.a.). On the basis of our assumption that tamoxifen suppressed GH levels, the results suggest that the lower GH levels in the tamoxifen-treated rats did not affect the expression of CYP2C11. Previous studies involving dwarf rats (Bullock et al., 1991) and adult rats treated neonatally with 2 mg MSG/g body weight (Pampori et al., 1991) have demonstrated that very low levels of GH are capable of maintaining normal sexual differentiation of CYP2C11 expression and its enzyme activities as long as the characteristic male GH secretory pattern remains. It is only upon the complete elimination of circulating GH via hypophysectomy (Waxman et al., 1991) or neonatal treatment with 4 mg MSG/g body weight (Pampori et al., 1991; Agrawal et al., 1991) that the sex difference is abolished. Hepatic microsomal testosterone 6β - and 7α -hydroxylase activities were also not significantly affected by tamoxifen treatment in adult male rats. In contrast, microsomal testosterone 6β- and 7α -hydroxylase activities were significantly altered in adult female rats. The lack of a significant effect of tamoxifen on any of the catalytic activities measured in male rats suggests that the changes in the catalytic activities observed in tamoxifen-treated female rats are sex-related and are likely hormone-mediated. Testosterone 6β-hydroxylase activity was increased in female but not male rats, while testosterone 7α -hydroxylase activity

was decreased in female but not male rats treated with the same dose of tamoxifen. Therefore, the changes cannot be as a result of induction or inhibition, respectively, by tamoxifen or one of its metabolites.

PROD activity was monitored in hepatic microsomes prepared from tamoxifentreated female rats because White *et al.* (1992) had previously demonstrated a 60-fold increase in this activity in hepatic microsomes prepared from adult female rats treated with large doses of tamoxifen (45 mg/kg *i.p.*) for four days and killed 24 hours later. These investigators most likely saw a direct inductive effect of tamoxifen itself on cytochromes P450. As we are interested in the influence of tamoxifen via its long term hormonal effects, we wanted to verify that any direct (i.e. inductive) effects of tamoxifen did not persist up to five weeks after treatment. The results demonstrate that tamoxifen treatment did not have a significant effect on hepatic microsomal PROD activity. It should be noted that the PROD activity observed with tamoxifen-treated and vehicle-treated female rats was at or near baseline, indicating that the CYP2B isozymes were not induced. This lack of induction of CYP2B1/CYP2B2 suggests that we were only observing the effects of tamoxifen indirectly through its hormonal alterations and not as a direct effect of tamoxifen itself.

In summary, hepatic microsomal cytochrome P450 enzyme activites were affected by tamoxifen treatment in adult female rats. For the reasons discussed above, it appears that these effects are sex-related and the result of altered hormone levels.

4.5. COMPARISON OF THE EFFECTS OF TAMOXIFEN AND NEONATAL OVARIECTOMY ON 7α-TESTOSTERONE HYDROXYLATION AND CYTOCHROME P450 2A1 PROTEIN LEVELS

The major rat cytochrome P450 isozyme responsible for catalyzing testosterone 7α hydroxylase activity is CYP2A1 (Levin *et al.*, 1987). Sonderfan *et al.* (1987) showed that purified rat liver microsomal CYP2A1 catalyzed the 7α -hydroxylation of testosterone and that this metabolite was not further hydroxylated during a 60 minute incubation of testosterone with reconstituted CYP2A1. Prior to Sonderfan's study, Wood *et al.* (1983) and Levin *et al.* (1987) had used antibody inhibition studies to show that antibody against CYP2A1 inhibits greater than 95% of the testosterone 7α -hydroxylase activity found in rat liver microsomes. These studies provide evidence for the inherent association between testosterone 7α -hydroxylase catalytic activity and CYP2A1 protein level.

CYP2A1 is known to be female-predominant in rat liver, as it is expressed at a higher level in female rats than in male rats of the same age (Thomas *et al.*, 1981; Waxman *et al.*, 1985; Sonderfan *et al.*, 1987; Waxman *et al.*, 1989). In the tamoxifen study, hepatic microsomal 7 α -hydroxylase activity was 7.6-fold greater in the vehicle-treated female rats than in the vehicle-treated male rats (Table 3.4.a.). This sex difference is similar to that reported by Sonderfan *et al.* (1987) and Waxman *et al.* (1989) of a 3.5- and 4.8-fold greater hepatic microsomal 7 α -hydroxylase activity in adult female rats versus adult male rats, respectively. CYP2A1 is also known to be age-dependent, as studies have shown that levels of this protein are decreased from about two to eight weeks of age in male rats with female rats showing much less of a decline (Waxman *et al.*, 1985; Nagata *et al.*, 1987). Waxman *et*

al. (1989) proposed that this developmental suppression of CYP2A1 in male rats is due to the increased serum GH levels characteristic of the postsuckling period. This age-related decline is not observed in female rats because CYP2A1 is believed to be positively regulated by the more continuous serum GH profile found in adult female rats.

In the present study, hepatic microsomes prepared from the tamoxifen-treated female rats had significantly less testosterone 7a-hydroxylase activity than microsomes prepared from the vehicle-treated females (57.9% of the mean activity of the latter group, see Table The decrease in testosterone 7α -hydroxylase activity is consistent with the 3.4.a.). significantly lower CYP2A1 protein levels observed in hepatic microsomes prepared from the tamoxifen-treated female rats as compared to those from the vehicle-treated female rats (70.7% of the mean CYP2A1 content of the latter group, see Table 3.7.a.). This decrease cannot be explained by the tamoxifen-induced suppression of GH levels as female rats hypophysectomized during adulthood neither show a significant difference in hepatic microsomal testosterone 7 α -hydroxylase activity (Waxman *et al.*, 1989) nor in hepatic microsomal CYP2A1 levels (Waxman et al., 1989; Yamazoe et al., 1990) from that of normal adult female rats. As with hypophysectomy, depletion of serum GH to undetectable levels (less than 2 ng/mL) by neonatal administration of MSG did not significantly affect hepatic CYP2A1 levels in adult female rats (Waxman et al., 1990). A study that used dwarf rats (Bullock *et al.*, 1991) also showed that hepatic microsomal testosterone 7α -hydroxylase activity and CYP2A1 protein levels of female dwarf rats, which have circulating GH levels of less than 5 ng/mL, were comparable to those of our vehicle-treated adult female rats. Our results are consistent with the conclusion made by Waxman et al. (1989) that although

CYP2A1 can be stimulated by continuous plasma GH secretion, its expression is not obligatorily dependent on continuous plasma GH. Therefore, tamoxifen may be exerting an influence on CYP2A1 expression through a mechanism other than its effects on GH levels.

Like the female tamoxifen-treated rats, hepatic microsomes prepared from the neonatally ovariectomized rats in this study (with and without estradiol treatment) had less microsomal testosterone 7α -hydroxylase activity than microsomes prepared from the appropriate neonatally sham-operated rats (approximately 76% of the activity of the shamoperated groups), but this effect was not statistically significant (p>0.05) (Table 3.4.b.). However, the CYP2A1 level was significantly lower in microsomes prepared from the neonatally ovariectomized rats as compared to those from the sham-operated rats (82.7% of the CYP2A1 content of the latter group, see Table 3.7.b.). Waxman et al. (1989) also investigated the effect of neonatal ovariectomy on hepatic microsomal testosterone 7α hydroxylase activity and CYP2A1 protein levels in the adult rat. In agreement with our results, they found testosterone 7 α -hydroxylase activity and CYP2A1 levels to be decreased in microsomes prepared from neonatally ovariectomized rats as compared to those from control female rats (approximately 70% of the levels of the latter group) (no statistical analyses done). As discussed previously, neonatal ovariectomy has been shown to decrease mean serum estradiol levels in the adult female rat (Jansson and Frohman, 1987b), but the serum estradiol levels in the neonatally ovariectomized rats in the present study were not significantly different from those of the sham-operated rats. Neonatal ovariectomy has also been shown to decrease mean plasma GH levels slightly, yet significantly, in adult female rats (Jansson and Frohman, 1987b).

Microsomal testosterone 7α -hydroxylase activities were not significantly different for rats that were administered estradiol during adulthood relative to rats that were not administered estradiol (Table 3.4.b.). However, the mean microsomal CYP2A1 levels of the estradiol-treated rats were significantly higher (approximately 1.5-fold greater) than those of the appropriate groups not administered estradiol (Table 3.7.b.). Waxman et al. (1989) also treated two groups of neonatally ovariectomized rats with estradiol, both groups received an estradiol packed capsule implanted at five weeks of age and one group also received estradiol benzoate (150 μ g, s.c.) on days one and three of life. Microsomal 7 α -hydroxylase activity was 1.3-fold greater in rats that received estradiol at five weeks of age only, while in the group administered estradiol both neonatally and at five weeks of age, microsomal 7α hydroxylase activity was 1.5-fold greater than that of neonatally ovariectomized rats, but no statistical analyses were performed. The immunoquantitation data revealed that only the rats that received both the estradiol capsule and the neonatal estradiol had significantly higher microsomal CYP2A1 levels than the neonatally ovariectomized rats (Waxman et al., 1989). These investigators concluded that estrogen makes a positive contribution to the expression of CYP2A1. Our data supports this conclusion since the estradiol-treated rats in our study had significantly higher microsomal CYP2A1 levels than the rats not administered estradiol. and the serum estradiol levels were approximately 20-fold higher in the rats administered estradiol during adulthood (Table 3.2.b.) as compared to the rats that did not receive this treatment.

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In summary, the results of the present study can be interpreted as follows: (i) decreased hepatic expression of CYP2A1 in tamoxifen-treated adult female rats was not

likely due to suppression of GH secretion, (ii) neonatal ovariectomy had an effect similar to that of tamoxifen treatment on hepatic CYP2A1 expression without a significant effect on serum estradiol levels, and (iii) administration of estradiol increased the expression of hepatic CYP2A1 in adult female rat liver. Thus, it appears that the expression of hepatic CYP2A1 is enhanced by estradiol, and that tamoxifen treatment and ovariectomy both decrease CYP2A1 levels, possibly through the same mechanism, but this mechanism is unknown.

4.6. COMPARISON OF THE EFFECTS OF TAMOXIFEN AND NEONATAL OVARIECTOMY ON 6β-TESTOSTERONE HYDROXYLATION AND CYTOCHROME P450 3A PROTEIN LEVELS

The major cytochrome P450 isozymes responsible for catalyzing testosterone 6β -hydroxylation in rat liver microsomes belong to the CYP3A subfamily (Waxman *et al.*, 1985; Sonderfan *et al.*, 1987; Halvorson *et al.*, 1990; Cooper *et al.*, 1993). Antibody-inhibition studies have demonstrated that polyclonal antibody against CYP3A inhibited greater than 85% of testosterone 6β -hydroxylase activity in liver microsomes from adult male rats (Waxman *et al.*, 1985; Halvorson *et al.*, 1990). However, it is unclear as to how many members of the CYP3A subfamily exist in rats and which forms are responsible for catalyzing the 6β -hydroxylation of testosterone. Sonderfan *et al.* (1987) showed that testosterone 6β -hydroxylation was catalyzed by a CYP3A protein that was partially purified from adult male rats induced with troleandomycin, purified CYP1A1, and purified CYP2C11 at turnover rates of 3.1, 1.5, and 0.35 nmol/nmol cytochrome P450/min, respectively. In untreated rats the level of CYP1A1 is low (Thomas *et al.*, 1981; Parkinson *et al.*, 1983b;

Waxman et al., 1985), which is consistent with observations made by Wood et al. (1983) that CYP1A1 contributes negligibly to testosterone 6^β-hydroxylase activity in rat liver. The small turnover rate measured for purified CYP2C11 suggests that this isozyme plays a minor role in the overall hepatic microsomal testosterone 6β-hydroxylase activity in adult male rats. Furthermore, CYP2C11 does not contribute to this catalytic activity in female rats as it is a male-specific isozyme. Additional evidence that hepatic microsomal testosterone 6βhydroxylation is catalyzed predominantly by CYP3A includes the fact that the age- and sexdependence of testosterone 6B-hydroxylase activity parallels that of CYP3A protein levels (Waxman et al., 1985) and testosterone 6^β-hydroxylase activity is dramatically increased (from 4- to 31-fold) by treatment of rats with pregnenolone- 16α -carbonitrile, which is a known CYP3A inducer (Sonderfan et al., 1987). CYP3A is also believed to catalyze 2β-, 15β-, and 18-hydroxylation of testosterone as minor pathways (Waxman *et al.*, 1985; Sonderfan et al., 1987). Recently, Cooper et al. (1993) isolated monoclonal antibodies that are isozyme-specific for CYP3A1 or CYP3A2. Prior to their study, polyclonal antibodies and monoclonal antibodies were not able to discriminate between these two isozymes. Cooper et al. (1993) reported that CYP3A1 is not detectable in untreated male or female rats of any age and that CYP3A2 is present in untreated adult male and untreated immature male and female rats, but not adult female rats.

Testosterone 6β -hydroxylase activity is present at similar levels in hepatic microsomes prepared from immature male and female rats and mature male rats, but is substantially decreased in hepatic microsomes prepared from adult female rats (Sonderfan *et al.*, 1987). In the present study, hepatic microsomal testosterone 6β -hydroxylase activity was

5.8-fold greater in vehicle-treated male rats than in vehicle-treated female rats (Table 3.4.a.). This sex difference is similar to that reported by Sonderfan *et al.* (1987) of a 10.4-fold greater hepatic microsomal testosterone 6β -hydroxylase activity in adult male rats versus adult female rats. The age-dependent decline in testosterone 6β -hydroxylase activity in female rats is thought to be caused by the lower mean GH levels in these animals. Surprisingly, there is no detectable CYP3A1 nor CYP3A2 in untreated adult female rats, as indicated by our data, yet testosterone 6β -hydroxylase activity is easily measureable.

In the present study, hepatic microsomal testosterone 6β -hydroxylase activity in male rats that had been treated with tamoxifen was not significantly different from that of the vehicle-treated male rats. Yamazoe *et al.* (1986) showed that hypophysectomizing male rats at seven weeks of age increased hepatic microsomal testosterone 6β -hydroxylase activity and that administering human GH, either by intermittent injections or continuous infusion, to normal and hypophysectomized male rats decreased hepatic microsomal testosterone 6β -hydroxylase activity, indicating that GH suppresses testosterone 6β -hydroxylase activity. In contrast, Waxman *et al.* (1990) showed that loss of pulsatile GH secretion as a result of neonatal treatment with 4 mg MSG/g body weight suppressed hepatic microsomal testosterone 6β -hydroxylase activity and CYP3A protein levels. Bullock *et al.* (1991) reported that the catalytic activity of CYP3A in liver microsomal testosterone 6β -hydroxylase activity observed in the tamoxifen-treated male rats in the present study suggests that low GH levels are enough to sustain the expression of this enzyme. Also, the conflicting reports in the literature about the effects of loss of GH suggest that CYP3A is not regulated by GH alone.

In contrast to the results obtained with male rats in our study, tamoxifen-treated female rats had significantly greater (1.6-fold) testosterone 6β -hydroxylase activity than vehicle-treated females (Table 3.4.a.). The increase in testosterone 6β -hydroxylase activity with tamoxifen treatment is not consistent with the immunoquantitation data generated using a polyclonal antibody to CYP3A1, which showed no significant difference between microsomal CYP3A levels of the tamoxifen-treated female rats and those of the vehicle-treated female rats (Table 3.10.a.). Hypophysectomy has been shown to elevate the level of hepatic CYP3A more than 10- to 20-fold in adult female rats (Waxman *et al.*, 1988). Unlike hypophysectomy, loss of circulating GH as a result of neonatal treatment with 4 mg MSG/g body weight did not cause an increase in CYP3A levels (i.e. no expression) in adult female rats (Waxman *et al.*, 1990). Dwarf rats were reported as having approximately 50% of the hepatic microsomal testosterone 6β -hydroxylase activity and CYP3A levels of those of normal rats (Bullock *et al.*, 1991).

Unlike the female tamoxifen-treated rats, microsomal testosterone 6β -hydroxylase activity measured in the neonatally ovariectomized rats in this study, with and without estradiol treatment, did not vary significantly from that of the appropriate neonatally shamoperated group (Table 3.4.b.). Waxman *et al.* (1985) also reported that birth ovariectomy had no effect on testosterone 6β -hydroxylase activity. Estradiol treatment during adulthood did not have a significant effect on microsomal testosterone 6β -hydroxylase activity. However, microsomal CYP3A levels in the estradiol-treated rats were significantly lower than the levels in the rats not administered estradiol (approximately 24% of the mean CYP3A content of the latter group).

Monoclonal antibodies against CYP3A1 and CYP3A2 were used to probe blots containing hepatic microsomes from tamoxifen-treated adult female rats, vehicle-treated adult female rats, adult male rats, dexamethasone-treated adult female rats, and phenobarbital-treated adult male rats (not shown). As reported by Cooper *et al.* (1993), monoclonal antibody specific for rat CYP3A1 detected a single band in hepatic microsomes from dexamethasone-treated adult female rats and phenobarbital-treated adult female rats and phenobarbital-treated adult male rats only, and monoclonal antibody specific for rat CYP3A2 detected a single band in hepatic microsomes from untreated and phenobarbital-treated adult male rats only (results not shown in this thesis). The inability of the two monoclonal antibodies to recognize any protein in hepatic microsomes from adult female rats indicates that adult female rats do not express CYP3A1 or CYP3A2 and suggests that another CYP3A isozyme with catalytic activity for 6β-hydroxylation of testosterone must exist in adult female rat livers.

In summary, we can conclude that: (i) if treatment with tamoxifen produced a GHlowering effect, the decrease in the serum GH level in male rats was not sufficiently profound to result in an increase in hepatic microsomal testosterone 6β -hydroxylase activity, (ii) tamoxifen treatment significantly increased hepatic microsomal testosterone 6β -hydroxylase activity in adult female rats but without a significant change in the protein level of CYP3A suggesting that tamoxifen either activates testosterone 6β -hydroxylase activity or another cytochrome P450 isozyme, not belonging to the CYP3A subfamily, hydroxylates testosterone at the 6β -position, (iii) neonatal ovariectomy did not significantly affect hepatic microsomal

testosterone 6β -hydroxylase activity or CYP3A levels in adult female rats, while administration of estradiol during adulthood to both neonatally ovariectomized and intact female rats significantly decreased the expression of CYP3A in adult female rats suggesting that estradiol suppresses CYP3A expression, and (iv) CYP3A1 and CYP3A2 are not present in hepatic microsomes prepared from adult female rats. In many cases, there is a discrepancy between the effects of treatment on testosterone 6β -hydroxylase activity and on CYP3A protein levels. The fact that the polyclonal antibody for CYP3A detected a single band in all of the adult female rat hepatic microsomes, and that hepatic microsomes prepared from adult female rats displayed testosterone 6β -hydroxylase activity, while CYP3A1 and CYP3A2 were not detected in any of the hepatic microsomes prepared from adult female rats, suggests (as speculated by Cooper *et al.* (1993)), that a third, female-specific CYP3A isozyme exists and has not yet been identified. The existence of another isozyme in the CYP3A subfamily could account for the inconsistency between studies.

4.7. COMPARISON OF THE EFFECTS OF TAMOXIFEN AND NEONATAL OVARIECTOMY ON p-NITROPHENOL HYDROXYLATION AND CYTOCHROME P450 2E1 PROTEIN LEVELS

The hydroxylation of *p*-nitrophenol has been shown to be a good indicator of CYP2E1 levels in the rat, rabbit, and humans (Reinke and Moyer, 1985; Koop, 1986; Tassaneeyakul *et al.*, 1993). Koop (1986) demonstrated that 90% of *p*-nitrophenol hydroxylase activity of microsomes prepared from acetone- or ethanol-treated rats was inhibited with anti-3a IgG (a rabbit analogue of CYP2E1). *p*-Nitrophenol was preferred over

other CYP2E1 substrate probes because both aniline and paracetamol are oxidized by several other cytochrome P450 isoforms, and N-nitrosodimethylamine is a known carcinogen and radiolabelled compound is required to measure N-demethylase activity using this substrate (Tassaneeyakul *et al.*, 1993).

Tamoxifen treatment did not significantly affect hepatic microsomal *p*-nitrophenol hydroxylase activity in female rats as compared to vehicle-treated rats (Table 3.5.a.). Similarly, the immunoquantitation data shows no significant difference between microsomal CYP2E1 levels in the tamoxifen-treated and vehicle-treated female rats (Table 3.9.a.). On the basis of our assumption that tamoxifen suppressed GH levels the results suggest that the lower GH levels in the tamoxifen-treated rats did not affect the expression of CYP2E1. Removal of GH, however, has been reported to increase CYP2E1 levels in adult rats. Hypophysectomy has been shown to increase CYP2E1 levels in adult male and female rats 3-to 5-fold and this effect was reversed by GH administered either intermittently or continuously (Waxman *et al.*, 1989; Yamazoe *et al.*, 1989). Depletion of serum GH by neonatal MSG-treatment also resulted in a 2-fold elevation of hepatic CYP2E1 levels in adult rats of both sexes (Waxman *et al.*, 1990). Thus, it appears that GH suppresses hepatic CYP2E1 expression.

Like tamoxifen treatment, neonatal ovariectomy did not have a significant effect on hepatic microsomal *p*-nitrophenol hydroxylase activity (Table 3.5.b.) or CYP2E1 levels (Table 3.9.b.). Waxman *et al.* (1989) observed a significant increase in microsomal CYP2E1 levels in neonatally ovariectomized rats compared to those of normal adult female rats. This discrepancy may be due to the fact that the neonatally ovariectomized rats in our study did not have lower serum estradiol levels than the neonatally sham-operated rats, whereas other

studies have reported that neonatally ovariectomized rats have lower serum estradiol levels than normal adult female rats (Jansson and Frohman, 1987b). This explanation is supported by the hypothesis that expression of CYP2E1 is suppressed by estrogen (Waxman *et al.*, 1989).

The rats administered estradiol during adulthood had noticeably lower microsomal pnitrophenol hydroxylase activities than the rats not administered estradiol (78% of the activity of the latter group) (Table 3.5.b.), but this difference was not statistically significant (p>0.05). However, there was a larger difference in relative CYP2E1 levels, determined by densitometric quantitation, between rats administered estradiol and those not administered estradiol (Table 3.9.b.). The mean CYP2E1 level of the neonatally sham-operated rats administered estradiol during adulthood was 61.5% of the level of CYP2E1 in sham-operated rats not administered estradiol, and neonatally ovariectomized rats administered estradiol during adulthood had only 52.9% of the microsomal CYP2E1 level of the ovariectomized rats not administered estradiol, with the latter difference being statistically significant. Waxman *et al.* (1989) reported that the increase in microsomal CYP2E1 levels in neonatally ovariectomized rats was reversed by estradiol treatment given either at five weeks of age or both neonatally and at five weeks of age. The findings of Waxman's study support our results in that estradiol appears to suppress the expression of CYP2E1.

In summary, we can conclude that: (i) the GH-lowering effects of tamoxifen treatment did not affect hepatic microsomal *p*-nitrophenol hydroxylase activity and CYP2E1 expression in adult female rats, (ii) neonatal ovariectomy did not affect hepatic microsomal *p*-nitrophenol hydroxylase activity or CYP2E1 levels in adult female rats because there was no change in serum estradiol levels, and (iii) estradiol administration during adulthood suppressed hepatic microsomal *p*-nitrophenol hydroxylase activity and CYP2E1 expression in adult female rats. These results suggest that the antiestrogenic activity of tamoxifen is not present five weeks after administration because we would have expected to see an increase in CYP2E1 levels if estradiol activity was blocked.

4.8. COMPARISON OF THE EFFECTS OF TAMOXIFEN AND NEONATAL OVARIECTOMY ON CYTOCHROME P450 2C7 PROTEIN LEVELS

CYP2C7 could not be monitored catalytically in liver microsomes because this isozyme does not have an easily measured, characteristic, microsomal activity (Waxman, 1991). Expression of CYP2C7 is age-dependent, as it has been shown that the hepatic microsomal concentration of CYP2C7 is low in immature rats and increases markedly during puberty to reach adult levels by seven weeks of age (Bandiera *et al.*, 1986). It is also sex-dependent, as it has been shown that the hepatic level of CYP2C7 is about 2-fold greater in adult female rats than in adult male rats (Bandiera *et al.*, 1986).

Tamoxifen treatment of adult female rats did not affect hepatic microsomal CYP2C7 levels significantly (Table 3.8.a.). Female rats hypophysectomized at six weeks of age and sacrificed at eight weeks of age showed a significant decrease in hepatic CYP2C7 mRNA levels (Westin *et al.*, 1990). Continuous administration of GH to hypophysectomized rats for six days restored CYP2C7 mRNA levels to those of normal adult female rats, while discontinuous GH treatment (male-specific pattern) reduced CYP2C7 expression further. The fact that treatment with tamoxifen did not significantly affect the expression CYP2C7 in liver suggests that if GH levels were suppressed in tamoxifen-treated rats then this isozyme only requires a minimal level of GH for its full expression. In contrast to the results obtained with the tamoxifen-treated female rats, hepatic microsomes prepared from neonatally ovariectomized rats had significantly lower CYP2C7 levels than those from neonatally sham-operated rats (26.7% of the CYP2C7 level of the latter) (Table 3.8.b.). However, there was no significant difference in microsomal CYP2C7 levels between the neonatally ovariectomized rats treated with estradiol during adulthood and sham-operated rats treated with estradiol during adulthood (Table 3.8.b.). The same results were reported in a previous study that used competitive ELISA for immunoquantitiation (Bandiera and Dworschak, 1992). Thus, our findings support the conclusion made by Bandiera and Dworschak (1992) that CYP2C7 is maximally expressed in intact female rats and that this expression cannot be further increased by estradiol treatment, whereas in ovariectomized female rats estradiol administration can increase the expression of CYP2C7 up to normal levels.

In summary, we can conclude that: (i) tamoxifen treatment did not affect hepatic microsomal CYP2C7 levels in adult female rats indicating that low GH levels are sufficient for the full expression of this isozyme, (ii) hepatic microsomal CYP2C7 expression was decreased as a result of neonatal ovariectomy but can be restored by treatment with estradiol during adulthood suggesting that estradiol induces CYP2C7, and (iii) hepatic CYP2C7 appears to be maximally expressed in intact female rats and cannot be increased further by estradiol treatment. The results suggest that the antiestrogenic activity of tamoxifen is not present five weeks after dosing because we would have expected to see a decrease in the expression of CYP2C7 if estradiol's effects were blocked.

4.9. CONCLUSIONS

In conclusion, the results of the present study verify our hypothesis, that tamoxifen alters the levels of hepatic cytochrome P450 isozymes that are hormonally regulated by either GH or estradiol. Once again, it must be emphasized that the regulation of cytochromes P450 is very complex and that each individual isozyme is uniquely influenced by changes in hormone levels. The fact that tamoxifen treatment did not affect the hepatic expression of CYP2E1 and CYP2C7 in adult female rats suggests that: (i) the GH-lowering effects of tamoxifen were not drastic enough to alter the normal expression of these isozymes (whereas CYP2E1 and CYP2C7 have been shown to be increased and decreased, respectively, by hypophysectomy) and (ii) the antiestrogenic effect of tamoxifen was not present in the liver five weeks after administration, because if estradiol's effects were blocked we would have expected to see an increase in CYP2E1 and a decrease in CYP2C7 levels.

The expression of CYP2A1, along with its characteristic testosterone 7α -hydroxylase activity, was decreased in livers of adult female rats treated with tamoxifen. Estradiol enhanced CYP2A1 expression but the antiestrogenic activity of tamoxifen does not appear responsible for the suppression of this isozyme because this acitivity does not seem to be present at this timepoint (as discussed for CYP2E1 and CYP2C7). The effect elicited by tamoxifen is similar to that observed in the ovariectomized rats whose estradiol levels were comparable to those of the sham-operated rats. This suggests that there is a common, yet unknown, factor altered by tamoxifen treatment and ovariectomy that results in decreased expression of hepatic CYP2A1.

Tamoxifen treatment increased hepatic testosterone 6β-hydroxylase activity in adult female rats but this increase was not reflected in CYP3A protein levels. Moreover, it was determined that CYP3A1 and CYP3A2 were not expressed in tamoxifen-treated female rats. A possible explanation of our results is that tamoxifen or a metabolite is activating CYP3Amediated enzyme activity without inducing the protein. An alternate explanation is that a novel isozyme, distinct from CYP3A1 and CYP3A2 and not recognized by our polyclonal antibody to CYP3A1, is induced in the tamoxifen-treated female rats but not in the tamoxifen-treated male rats. Although the CYP3A subfamily has been extensively characterized by several research groups it is possible that all of the isozymes in this family have not yet been identified. Overall, this study has established some interesting observations which serve as a basis for many, as of yet, unanswered questions.

4.10. FUTURE STUDIES

4.10.1. Antibody inhibition experiments

CYP3A is thought to be the main isozyme responsible for testosterone 6β -hydroxylation (Waxman *et al.*, 1985; Sonderfan *et al.*, 1987; Nagata *et al.*, 1989). The conclusions drawn from the above study assume that testosterone 6β -hydroxylase activity correlates with CYP3A levels in the hepatic microsomes prepared from the tamoxifen-treated adult female rats. To ensure that this association is correct, the testosterone hydroxylase assay should be performed in the presence of one or more antibodies against CYP3A. If a CYP3A-related isozyme is responsible for hydroxylation of testosterone at the 6β -position, the presence of anti-CYP3A should inhibit this reaction.

4.10.2. Role of GH and estradiol in tamoxifen-induced alterations of hepatic cytochromes *P450*

As demonstrated in this thesis, the regulation of cytochromes P450 is extremely complex and it is very difficult to establish any firm conclusions. To help verify which hormones are responsible for the effects observed on hepatic cytochromes P450 after tamoxifen treatment, it would be useful to minimize the number of hormones being altered. Since GH and estradiol are believed to be the two main hormones affected by tamoxifen, artificially increasing either GH or estradiol levels independently and determining if the cytochrome P450 profile changes would give more insight into the mechanism through which tamoxifen is exerting its effects.

To determine if suppression of GH secretion is responsible for all of the effects of tamoxifen on hepatic cytochromes P450, the experiment could be repeated while increasing the GH levels after tamoxifen treatment by administering exogenous GH. If all cytochrome P450 isozymes affected by tamoxifen treatment return to their pre-treatment levels after administration of exogenous GH, it would indicate that GH is the primary controlling factor in their regulation. However, if some isozymes do not respond to manipulation of the GH secretory pattern, it would suggest that they may be under the influence of other hormonal factors.

To determine the effects of estradiol (independent of GH), the experiment could be repeated with the administration of a synthetic GH-releasing factor antagonist (to block GH secretion) along with the administration of a supraphysiological dose of estradiol daily. A larger dose of estradiol is required to displace tamoxifen or its metabolites from estrogen binding sites. Any cytochrome P450 isozymes affected by tamoxifen treatment that return to

their pre-treatment levels after the administration of exogenous estradiol are most likely primarily regulated by estradiol.

4.10.3. Time-course and dose-response of the effect of tamoxifen on hepatic cytochromes P450

The study completed for this thesis found that adult female rats injected with 5 mg of tamoxifen once daily for two consecutive days had significantly altered hepatic cytochrome P450-mediated catalytic activities and protein levels. White *et al.* (1992) saw a different hepatic cytochrome P450 profile using much greater doses of tamoxifen and much shorter incubation times. Thus, it would be interesting to observe how the cytochrome P450 pattern of expression varies with the length of time after administration, and the dose, of tamoxifen.

The effect of tamoxifen (5 mg *s.c.* once daily for two consecutive days) on GH secretion has been shown by Tannenbaum *et al.* (1992) to vary between 18 hours, one to two weeks, and three to seven weeks after administration. It would therefore be interesting to examine how the expression of specific cytochromes P450 changes during this time period. Also, it is important to study the effects of tamoxifen on cytochromes P450 beyond seven weeks to determine how persistent the effects of tamoxifen and its metabolites are.

The dose of tamoxifen used in this thesis is equivalent to approximately 20 mg/kg body weight (13.5 mol/kg body weight) which is much larger than the daily dosage of 20 mg used for breast cancer therapy. To make this study more clinically relevant it would be beneficial to determine if smaller doses of tamoxifen cause a similar suppression of circulating GH levels in rats and if hepatic cytochrome P450 expression is affected by lower tamoxifen doses. Also, it would be interesting to investigate whether larger doses of

tamoxifen would cause more pronounced, or different, effects on the pattern of cytochrome P450 expression and to determine the threshold dose where effects level off.

Tamoxifen is cytostatic and not cytotoxic. Therefore, tamoxifen therapy is a longterm process. It would be useful to investigate the effects of long-term tamoxifen treatment by administering tamoxifen daily for six months at a dose that best approximates the daily dosage for humans on tamoxifen therapy (0.5 mg/kg body weight).

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