THE INFLUENCE OF PERIPUBERTAL TESTOSTERONE ON HEPATIC MICROSOMAL ERYTHROMYCIN DEMETHYLASE IN PREPUBERTALLY OVARIECTOMIZED FEMALE RATS

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Division of Pharmacology and Toxicology of the Faculty of

Pharmaceutical Sciences)

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

November 1989

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Date   DECEMBER 23, 1989

ABSTRACT

The influence of peripubertal exposure to physiological levels of testosterone on the adult androgen responsiveness of the cytochrome P450 enzyme activity, hepatic microsomal erythromycin demethylase activity, was investigated.

Rats were injected subcutaneously with testosterone enanthate 5 μmoles/kg/day either peripubertally, during adulthood or in both time periods. In adult untreated rats, hepatic microsomal erythromycin demethylase activity was higher in males than in females. Intact adult male rats, but not intact adult female rats, responded to adult testosterone treatment with an increase in hepatic microsomal erythromycin demethylase activity.

Female and male rats were gonadectomized before the onset of puberty. In the adult female rats which had been prepubertally ovariectomized, exposure to testosterone peripubertally resulted in an adult androgen responsiveness for hepatic microsomal erythromycin demethylase activity. This indicated that the potential is present in the prepubertally ovariectomized female rat for the pubertal imprinting by testosterone of an adult androgen responsiveness for hepatic microsomal erythromycin demethylase activity.

Prepubertal castration of male rats reduced hepatic microsomal erythromycin demethylase activity and plasma testosterone levels from control levels. Hepatic microsomal erythromycin demethylase activity was found to be partially correlated with plasma testosterone levels. The higher hepatic microsomal erythromycin demethylase activity in the adult male rat may therefore be related to high adult male levels of circulating testosterone. The administration of testosterone to adult male rats which had been prepubertally castrated resulted in hepatic microsomal erythromycin demethylase activity which was lower than that of intact males and of intact males treated with testosterone in adulthood. These results indicated that adult androgen responsiveness of hepatic microsomal erythromycin demethylase activity is not completely imprinted in male rats in the neonatal period.

This study provided evidence in support of the hypothesis that the peripubertal period is a time during which imprinting by testosterone of adult androgen responsiveness of hepatic P450 enzymes can occur.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BPH	Benzo[a]pyrene hydroxylase
Dex	Dexamethasone
EDTA	Ethylenediamine-tetraacetic acid
HPLC	High performance (pressure) liquid chromatography
K	Potassium
3-MC	3-methylcholanthrene
Na	Sodium
NADPH	β-nicotinamide adenine-dinucleotide phosphate, reduced
Pb	Phenobarbital
PCN	Pregnenolone-16α-carbonitrile

ACKNOWLEDGEMENTS

This thesis is dedicated to Paul Warwick, who endured so much, and survived.

1. INTRODUCTION

The term cytochrome P450 refers to a collection of hemoprotein enzymes which, when reduced, form a complex with carbon monoxide with absorption peaks at approximately 450 nm (Omura & Sato, 1962); the "P" stands for pigment (Garfinkel, 1958; Klingenberg, 1958). These enzymes † are of scientific interest due to their ability to catalyze the biotransformation of both endogenous compounds, notably steroids, fatty acids and prostaglandins, as well as xenobiotics, including therapeutically-active drugs and potentially toxic chemicals. Although our knowledge of the cytochrome P450 enzymes in humans is rapidly expanding, the majority of studies in this area have used the rat model. Cytochrome P450 is ubiquitous, the highest concentration being found in liver cell endosplasmic reticulum (Guengerich, 1987). Therefore most studies, including the one presented here, investigate cytochrome P450 in microsomes prepared from rat liver.

It is currently known that the rat hepatic P450 family contains at least 20 different enzymes (Nebert *et al.*, 1987; Guengerich, 1987). The degree to which each of these P450 enzymes is expressed varies between individual animals,

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†The term isozyme is not used, since these enzymes catalyze different reactions.

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depending on genetic predisposition, for example a genetic polymorphism; on environmental factors, such as exposure to inducing chemicals; and on physiological factors such as sex, age and disease (Gonzalez, 1989). Research is currently directed at discovering the factors and mechanisms involved in the regulation of the expression of the different P450 enzymes. The observed sexual and developmental variations suggest that for certain P450s, in particular those which are present in untreated rats, regulation may involve hormones.

This study investigates, in simplest terms, the relationship between exposure to androgenic hormone during a discrete developmental period and a catalytic activity representative of P450 enzyme expression. As background, it will first be necessary to review briefly the theoretical framework within which such research has been conducted in the past.

1.1. THEORY OF ENDOCRINE CONTROL OF HEPATIC MICROSOMAL CYTOCHROME P450

1.1.1. Gustafsson's Theory

It has been known for some time that the rat liver is a sexually dimorphic organ (Roy & Chatterjee, 1983). Sexual differences exist in liver prolactin receptor levels (Kelly

et al., 1974), in the hepatic synthesis of urinary male-specific a2-microglobulin (Roy *et al.*, 1983), and in liver enzymes involved in the metabolism of drugs and steroids (Yates *et al.*, 1958). That hormones are involved in the control of hepatic steroid metabolism has been evident for at least 30 years. It was observed that neonatal male castration could remove sex differences in steroid metabolism whereas androgen treatment returned the activity to the normal male level (Yates *et al.*, 1958; Denef & DeMoor, 1968a, 1968b, 1972). The timing of these manipulations was also important; cortisol metabolism in male rats was "feminized" by neonatal gonadectomy but relatively unaffected by postpubertal castration (Denef & DeMoor, 1968a, 1968b, 1972). Einarsson, Gustafsson and Stenberg (1973) extended this work and formulated a theory relating sex hormones and hepatic microsomal metabolism. Based on their studies of the effects of castration or testosterone treatment at various developmental times (Berg & Gustafsson, 1973; Einarsson *et al.*, 1973), they postulated that microsomal enzymes could be grouped into at least three categories (Figure 1A, page 25). Group I enzymes, represented by 2a-hydroxylation of 4-pregnene-3,20-dione and 5a-androstane-3a,17β-diol, were entirely dependent on androgens for irreversible "imprinting" or "programming" prepubertally and reversible stimulation by androgens

postpubertally. Group II, exemplified by 6β-hydroxylation of 4-androstene-3,17-dione and 4-pregnene-3,20-diol, were only partially dependent on androgen, having a basal level of activity determined by nongonadal factors, as seen in females, but a reversible inducibility by androgens. The distinction between Groups I and II thus lay in the presence of Group II enzyme activity in females, and could be shown by the effects of neonatal castration of the male, which abolished Group I levels but did not abolish a Group II basal level. Group III enzymes, for example 7α-hydroxylation of 4-androstene-3,17-dione and cholesterol, were not dependent on androgens, being unaffected by gonadectomies and testosterone treatment.

Two important concepts were thus introduced. "Adult androgen responsiveness" was described as a reversible stimulation by androgens in the adult period. The word "imprinting" derived from its use by Konrad Lorenz (1971) within a behavioral context. As described by Lorenz (1971), "imprinting" involved an irreversible process which was limited to a "circumscribed ontogenetic phase". It has since been applied to other biological phenomena (Skett, 1979). Gustafsson applied "imprinting" to hepatic steroid metabolism as an effect of *neonatal* androgen on enzyme level and androgen responsiveness of the enzyme in the adult period (Gustafsson

& Stenberg, 1973; Gustafsson *et al.*, 1974a, 1974b). Therefore, according to the original theory, adult androgen responsiveness in Group I and II enzymes was imprinted neonatally, and in Group I a basal level was also imprinted neonatally by androgens (Gustafsson *et al.*, 1983). Gustafsson and coworkers observed that the 2α-hydroxylation of 5α-androstene-3α-17β-diol and the 6β-hydroxylation of 4-androstene-3,17-dione responded to adult testosterone in male rats castrated at 14 days of age, but not in neonatally castrated males (Gustafsson & Stenberg, 1974b), indicating imprinting of adult androgen responsivity within the first 14 days of life.

1.1.2. The Evolution of Gustafsson's Theory

Since 1973, our understanding of the endocrine control of hepatic steroid metabolism has benefited from several directions of research.

1.1.2.1. Multiple Hepatic Cytochrome P450 Enzymes

It should be realized that when Gustafsson's theory was first proposed in 1973, it was not widely accepted that hepatic P450 was composed of more than one enzyme. Indeed, at that time the idea that "the commonly-used term cytochrome P450 may actually embrace a group of cytochromes

with identical hemes bound to different proteins" was a matter of controversy (Einarsson *et al.*, 1973). It is now clear that rat hepatic P450 consists of multiple enzymes (Gonzalez, 1989), and there has been a great deal of research on the expression of specific P450 enzymes in untreated male and female rats. Tables I, II and III summarize current information regarding the levels of P450 enzymes in hepatic microsomes from untreated rats, and their responses to hormonal manipulation. Only those forms shown to be distinct enzymes on the basis of their spectral activity, electrophoretic mobility, substrate specificity and amino-terminal sequences, and purified to electrophoretic homogeneity, are presented. For other forms detected in rat liver microsomes (for reviews see Guengerich, 1987; Gonzalez, 1989), only minimal information is available on influence by hormonal factors. For simplicity, the nomenclature of Ryan & Levin (Ryan *et al.*, 1979, 1980, 1982, 1984, 1985) will be used for the majority of P450 forms, however in some cases nomenclature based on P450 gene families (Nebert *et al.*, 1987, 1989) will be used.

Several generalizations are apparent from these studies of specific P450 enzymes in untreated adult rat liver (Tables I-III). Firstly, rat hepatic microsomal P450 enzymes can be divided into those detected only in females or at greater

levels in females than in males (P450a, P450f, P450i), those detected only in males or at greater levels in adult males than females (P450g, P450h, RLM2 and the P450 III family), and those present at approximately equivalent levels (defined here as a less than two-fold difference) in untreated adults of both sexes (P450b, P450c, P450d, P450e, P450j, db1 and P450k). Secondly, the sexually differentiated P450 enzymes do not appear to be coordinately regulated, as indicated by their varied responses to gonadectomy, hypophysectomy and hormonal treatment. Rather, each sex-specific P450 appears to be regulated in a unique manner, and influenced by hormones. Thirdly, testosterone is oxidized in a regio- and stereo-selective manner which is characteristic of certain P450 forms. And fourthly, developmental changes are observed in the expression of many of the P450 enzymes, with many of the sex differences becoming manifest around puberty.

The present state of scientific investigation involves further work to identify the critical factors, in particular hormones, involved in the regulation of each specific P450, and to delineate the critical developmental time periods during which these factors exert their effects.

Table I. Predominantly Female P450 Forms in Liver Microsomes from Adult Rats. Animals were untreated, except as indicated. Values indicate immunoquantitated levels of protein, which may vary since different methods were used in different studies. Abbreviations: NC, no change; ⬆, increase; ⬇, decrease; Gx, gonadectomy; Hx, hypophysectomy; neo, neonatal; ND, not detectable; PR, partially restored to pre-surgical adult level; FR, fully restored to pre-surgical adult level; Pb, phenobarbital; 3-MC, 3-methylcholanthrene; βNF, βnaphthoflavone; Dex, dexamethasone; PCN, pregnenolone-16a-carbonitrile; Neg, negligible; T, testosterone.

References:
P450a: Jacobson & Kuntzman, 1969; Thomas *et al.*, 1981; Parkinson *et al.*, 1983; Waxman *et al.*, 1985; Dannan *et al.*, 1986; Nagata *et al.*, 1987; Arlotto & Parkinson, 1989; Arlotto *et al.*, 1989. P450f: Ryan *et al.*, 1984; Bandiera *et al.*, 1986. P450i: Kamataki *et al.*, 1983; MacGeoch *et al.*, 1984; Ryan *et al.*, 1984; Kamataki *et al.*, 1985; Waxman *et al.*, 1985.

	P450a	P450f	P450i
% of Total P450:			
Male:	3%	7%	ND
Female:	6%	14%	45%
Testosterone Hydroxylation Sites	$6a,7a$	$16a$	—
Developmental Changes:			
↑ week 1:male:	√	—	—
female:	√	—	—
↑ at puberty:male:	—	√	—
female:	—	√	√
↓ at puberty:male:	√	—	√
female:	—	—	—
Hormonal Effects:			
Gx_{neo}:male:	NC	—	↑
female:	↓50%	—	↓67%
Gx_{neo}+T:male:	—	—	ND
female:	—	—	—
Gx_{later}:male:	—	—	NC
female:	small↓	—	NC/↓
Gx_{later}+T:male:	—	—	—
female:	—	—	ND
Hx_{adult}:male:	—	—	ND
female:	—	—	ND
Thyroid Hormones:	↓	—	—
Inducers:Pb:	√	NC	NC
3-MC/βNF:	√	small↓	NC
Dex/PCN:	—	small↓	↑

Table II. Predominantly Male P450 Forms in Liver Microsomes from Adult Rats. Animals were untreated, except as indicated. Values indicate immunoquantitated levels of protein, which may vary since different methods were used in different studies. Abbreviations: NC, no change; $\blacktriangle$, increase; $\blacktriangledown$, decrease; Gx, gonadectomy; Hx, hypophysectomy; neo, neonatal; ND, not detectable; PR, partially restored to pre-surgical adult level; FR, fully restored to pre-surgical adult level; Pb, phenobarbital; 3-MC, 3-methylcholanthrene; βNF, βnaphthoflavone; Dex, dexamethasone; PCN, pregnenolone-16α-carbonitrile; Neg, negligible; T, testosterone; @, PCN2; †, PCN1; *, 2a/PCN-E; #, Pb-1.

References:
P450g: Cheng & Schenkman, 1982; 1983; Ryan *et al.*, 1984; Jansson *et al.*, 1985c; Bandiera *et al*, 1985; Bandiera *et al.*, 1986; McClellan-Green *et al.*, 1989. P450h: Cheng & Schenkman, 1982; 1983; Guengerich *et al.*, 1982; Kato & Kamataki, 1982; Kamataki *et al.*, 1983; Kamataki *et al.*, 1984; Ryan *et al.*, 1984; Waxman *et al.*, 1984; Kamataki *et al.*, 1985; Morgan *et al.*, 1985; Waxman *et al.*, 1985; Cresteil *et al.*, 1986; Dannan *et al.*, 1986; Kato *et al.*, 1986; Shimada *et al.*, 1987; Mode *et al.*, 1988; Shimada *et al.*, 1988. P450III: Gozukara *et al.*, 1984; Jansson *et al.*, 1985c; Waxman *et al.*, 1985; Cresteil *et al.*, 1986; Dannan *et al.*, 1986; Gonzalez *et al.*, 1986; Waxman *et al.*, 1986; Imaoka *et al.*, 1988; Yamazoe *et al.*, 1988. P450 RLM2: Jansson *et al.*, 1985a; Jansson *et al.*, 1985b; Jansson *et al.*, 1985c; Waxman *et al.*, 1986.

	P450g	P450h	P450RLM2	P450III
% of Total P450:				
Male:	<1%/>10%	33%		25%#
Female:	ND	ND	ND	<2%#
Testosterone Hydroxylation Sites	$6\beta,7a,15a$	$2a,2\beta,6\beta$ $16a,17a,$ $15-$	$6\beta,7a,$ $7\beta,15a$ 15β	6β#@
Developmental Changes:				
↑ in week 1:male:	–	–	–	√
female:	–	–	–	√
↑ at puberty:male:	√	√	√	√#@ NC*
female:	–	–	–	–
↓ at puberty:male:	–	–	–	–
female:	–	–	–	√
Hormonal Effects:				
Gx_{neo}:male:	ND	ND	↓	ND*
female:	–	–	–	–
Gx_{neo}+T:male:	PR	PR	–	PR*
female:	–	–	–	–
Gx_{later}:male:	NC	↓	–	NC*
female:	–	–	–	–
Gx_{later}+T:male:	–	FR	–	–
female:	–	PR	–	–
Hx_{adult}:male:	↑	↓	↑	↑
female:	↑	present	↑	↑
Thyroid Hormones:	–	NC	–	–
Inducers:Pb:	NC	↓	↓	↑@*#
3-MC/βNF:	small↓	↓	↓	–
Dex/PCN:	small↓	↓	↓	NC@ ↑*†

Table III. Approximately Equal P450 Forms in Liver Microsomes in Adult Male and Female Rats. Animals were untreated, except as indicated. Values indicate immunoquantitated levels of protein, which may vary, since different methods were used in different studies. Abbreviations: NC, no change; ⚡, increase; ⇂, decrease; Gx, gonadectomy; Hx, hypophysectomy; neo, neonatal; ND, not detectable; PR, partially restored to pre-surgical adult level; FR, fully restored to pre-surgical adult level; Pb, phenobarbital; 3-MC, 3-methylcholanthrene; βNF, βnaphthoflavone; Dex, dexamethasone; PCN, pregnenolone-16a-carbonitrile; Neg, negligible; T, testosterone.

References:
P450 b+e: Ryan *et al.*, 1979; Thomas *et al.*, 1981; Parkinson *et al.*, 1983, Waxman *et al.*, 1983; Wood *et al.*, 1983; Waxman *et al.*, 1985. P450 c+d: Thomas *et al.*, 1981; Parkinson *et al.*, 1983; Waxman *et al.*, 1985; Giachelli & Omiecinski, 1987. P450j: Song *et al.*, 1986; Thomas *et al.*, 1987; Williams & Simonet, 1988. P450k: Waxman & Walsh, 1983; Waxman *et al.*, 1985; Gonzalez *et al.*, 1986 P450 db1: Al-dabbagh *et al.*, 1981; Larrey *et al.*, 1984; Gonzalez *et al.*, 1987.

	P450b+e	P450c+d	P450j	P450k	P450db1
% of Total P450:					
Male:	1-2%	1%	<8%	-	6%
Female:	1-2%	1%	10%	-	0.3/6%
Testosterone Hydroxylation Sites	$16\alpha,16\beta$	$2\beta,6\beta$	-	$16\alpha,16\beta$	Neg
Developmental Changes:					
↑ in week 1:male:	-	-	√	-	√
female:	-	-	√	-	√
↑ at puberty:male:	-	-	-	√	√
female:	-	-	-	√	√
↓ at puberty:male:	√	√	√	-	-
female:	√	√	√	-	-
Hormonal Effects:					
Gx_{neo}:male:		NC	-	NC	-
female:	-	NC	-	small↓	-
Gx_{neo}+T:male:	-	NC	-	NC	-
female:	-	NC	-	NC	-
Gx_{later}:male:	-	-	-	-	-
female:	-	-	-	-	-
Gx_{later}+T:male:	-	-	-	-	-
female:	-	-	-	-	--
Hx_{adult}:male:	-	-	↑	-	-
female:	-	-	↑	-	-
Thyroid Hormones:	-	-	-	-	-
Inducers:Pb:	↑	-	-	↑	neg
3-MC/βNF:	-	↑	↓(male)	NC	neg
Dex/PCN:	↑	-	↓(male)	NC	neg

1.1.2.2. Sexual Differentiation of the Central Nervous System

Recent years have also brought an expansion of our knowledge concerning many sexually differentiated phenomona, including behavior and gonadotropin secretion. Several important conclusions have been reached.

As reviewed by MacLusky & Naftolin (1981), the general hypothesis for sexual differentiation of the CNS presumes that the CNS is intrinsically organized for the homogametic sex, this being females in mammals. A change away from this inherent pattern in males requires exposure to gonadal hormones (Gorski, 1971). In mammals, early gonadal secretions appear to be important. It is known that the sensitivity of the CNS to the permanent organizational effects of hormones varies with age. For rats, a "critical period" of CNS sexual differentiation of reproductive functions and sex behavior has been determined as approximately 18-27 days after conception, with birth usually occurring on days 20-22 (MacLusky & Naftolin, 1981).

Early hormonal effects on the CNS are usually permanent, and distinct from reversible hormonal effects observed in adulthood. In many animals the adult sex-related reproductive behavior depends on the presence of circulating hormone levels both during the early "critical period" and

also during later periods, since adult gonadectomy removes the behavior and hormone treatment restores it (Goy & McEwen, 1980; MacLusky & Naftolin, 1981).

In order to achieve masculine CNS functions two modification processes have been hypothesized as necessary: (i) a "defeminization" involving suppression of female patterns, and (ii) a "masculinization" which produces masculine characteristics (Beach, 1975).

Interestingly, it is currently thought that androgen acts on the developing rat brain via aromatization to estrogen and interaction with estrogen receptors. This is indicated by studies showing a block of neonatal testosterone effects by the estrogen antagonist MER-25 (McDonald & Doughty, 1972), and the demonstrated ability of the brain to convert testosterone to estrogen (Naftolin *et al.*, 1975). Endogenous estrogen does not enter the brain and masculinize female rats because it is bound in the circulation by α-fetoprotein, a product synthesized by the yolk sac and fetal liver which disappears over the first few weeks of life (Raynaud *et al.*, 1971; Uriel *et al.*, 1972; Aussel et al, 1973).

Neonatal female rats have significant levels of testosterone

in the plasma during the apparent "critical period" (Weisz &
Ward, 1980), and yet are obviously not masculinized. It is
possible that prior hormonal exposure has in some way
changed the response to subsequent testosterone (Weisz &
Ward, 1980). As well, it has been shown that the presence of
ovaries can inhibit defeminization of sexual behavior in
female rats by neonatal testosterone (Blizard & Denef,
1973).

Thus, current concepts of sexual differentiation of the CNS
are, as might be expected, similar to those related to
hepatic enzymes, including a "critical" imprinting period
and a response to adult hormones. It is not known if the
"critical" period is the same for all sexually
differentiated phonomena, such as liver enzymes.

1.1.2.3. The Hypothalamo- Pituitary- Liver Axis

Meanwhile, a relationship between the liver and the
hypothalamo-pituitary complex was becoming apparent. Early
studies by Denef (1974) and Gustafsson & Ingelman-Sundberg
(1975) had indicated a role for the pituitary gland in
sexually differentiated steroid metabolism. Hypophysectomy
removed the sex difference in Δ4-3-keto reduction of
testosterone (Denef, 1974) and in 15β-hydroxylase activity

(Gustafsson & Ingelman-Sundberg, 1975). Recently, the most markedly sex-specific P450 enzymes present in liver microsomes from noninduced rats, P450h and P450i (Tables I and II), have been shown to be influenced by the pattern of growth hormone secretion from the pituitary (MacGeoch *et al.*, 1984; Morgan *et al.*, 1985; MacGeoch *et al.*, 1987; Strom *et al.*, 1987).

In the rat, plasma growth hormone levels are high in the fetus and newborn (Rieutort *et al.*, 1974). Levels then decrease, to reach a low at days 18-22 of age. Growth hormone levels rise during the late prepubertal and pubertal period to attain a higher and sexually-differentiated pattern of secretion by 30 days of age (Edén, 1979). In the adult male rat, growth hormone is secreted from the pituitary in bursts at 3-4 hour intervals, and levels are low or undetectable between bursts (Edén, 1979). In the adult female, growth hormone secretion is more continuous, with higher basal levels and a lack of the high peaks of the adult male.

Using the male-specific P450h as an example, it has been shown that adult hypophysectomy reduces the normally high levels of P450h in male liver microsomes (Morgan *et al.*, 1985; Kamataki *et al.*, 1985). Administration of growth hormone by

continuous infusion, similar to the normal female pattern (Edén, 1979), reduces P450h in normal and hypophysectomized males (Kato *et al.*, 1986). Intermittent growth hormone, however, mimics the male secretion pattern and returns P450h and associated mRNA levels to those of a normal adult male in hypophysectomized males (Kato *et al.*, 1986; Shimada *et al.*, 1988; Mode *et al.*, 1988). Therefore, P450h expression in the adult male liver microsomes requires a pulsatile pattern of growth hormone secretion.

1.1.2.4. Androgenic Control of Growth Hormone Secretion Patterns
Concurrently, a connection has been established between gonadal steroids and the pattern of growth hormone secretion by the pituitary.

It has been shown that growth hormone secretion is under moment-to-moment control from the hypothalamus, with growth hormone releasing factor being stimulatory and somatostatin inhibitory (Brazeau *et al.*, 1973; Jansson *et al.*, 1985). The sexually differentiated pattern, however, has been shown by Jansson and co-workers (1984, 1985a, 1985b, 1985c, 1987), in an elegant series of experiments, to be organized by androgenic hormones. Neonatal castration, but not prepubertal castration, lowered the pulse height of growth hormone secretion in males (Jansson *et al.*, 1984). Females

treated with testosterone neonatally displayed typical high peaks of growth hormone secretion only if they were neonatally ovariectomized (Jansson & Frohman, 1987). Therefore, peak growth hormone levels were permanently set by neonatal testosterone, and the ovaries were inhibitory to this neonatal imprinting by testosterone. Both neonatal and prepubertal castration increased growth hormone baseline levels in male rats, an effect reversed by testosterone therapy (Jansson *et al.*, 1984). Therefore the typical low valleys in males appeared to be dependent on the continued presence of testosterone. In males treated with estrogen in adulthood, the basal growth hormone level was elevated, again suggesting a feminizing effect for estrogens which was antagonistic to the effects of testosterone (Mode *et al.*, 1982). Therefore, it currently appears that neonatal testosterone imprints a pattern of high growth hormone peaks in the male, and circulating testosterone in the male is required for the low basal level between peaks. Ovarian factors are inhibitory to both of these effects.

1.1.2.5. Current Theory

Given that gonadal hormones (Tables I and II) and growth hormone (Jansson *et al.*, 1983, 1984, 1985a, 1985b, 1985c) can influence the expression of certain hepatic microsomal P450 enzymes, it remained to be shown whether gonadal hormones

exert their effect by direct action on the liver, or indirectly by controlling growth hormone secretion patterns.

To date, a direct effect by androgens and estrogens on the liver remains a possibility. Androgen receptors are found in the liver, however a sex-related difference has not been shown (Gustafsson *et al.*, 1975). Estrogen receptors are also found in both male and female rat livers in approximately equal concentrations (Wrange *et al.*, 1980). However, the possibility does exist that one sex could possess another steroid-binding protein of high capacity and low affinity.

It has been shown that, for certain P450s at least, gonadal steroids may regulate their expression via effects on pituitary growth hormone. Consider again, for example, P450h. The most markedly male-specific P450 enzyme is P450h (Ryan *et al.*, 1984) which has been isolated by various groups as P450-male (Kamataki *et al.*, 1983), RLM5 (Cheng & Schenkman, 1982), P450-2c (Waxman & Walsh, 1983) and UT-A (Guengerich *et al.*, 1982). P450h has not been reported in microsomes from female rats of any age (Cheng & Schenkman, 1982; Kamataki *et al.*, 1983; Waxman *et al.*, 1984; Waxman *et al.*, 1985). P450h is strongly influenced by androgen, since castration of males at birth completely removed detectable levels in the adult (Kamataki *et al.*, 1983; Waxman *et al.*, 1985; Dannan *et al.*, 1986;

Shimada *et al.*, 1987). Partial male levels of expression of P450h in the neonatally castrated male could be produced by neonatal testosterone therapy (Kamataki *et al.*, 1983; Waxman *et al.*, 1985; Dannan *et al.*, 1986; Shimada *et al.*, 1987). Hypophysectomy also reduced the normally high P450h levels in adult male liver microsomes, an effect which was not reversed by testosterone therapy neonatally (Kamataki *et al.*, 1985; Kato *et al.*, 1986). Consistent with this was the observation that female rats, normally lacking P450h, would partially express P450h when hyphophysectomized, despite a relative lack of androgen (Kamataki *et al.*, 1985). These studies showed that, at least for P450h, growth hormone pattern is the main factor in sex-specific expression. These findings are consistent with the hypothesis that androgens influence P450s indirectly via growth hormone. These results were similar to those reported for the gonadal control of the female-specific P450i (MacGeoch *et al.*, 1984; Kamataki *et al.*, 1985): hypophysectomy of females removed the expression of P450i, while P450i expression was found in males treated with continuous growth hormone, mimicking the female pattern. Therefore, considerable evidence now exists for regulation of P450i and P450h by the gonadal-hypothalamo-pituitary-liver axis.

On the other hand, as can be seen from the varied effects of

hypophysectomy on the levels of the various P450 forms shown in Tables I and II, it is unlikely that identical regulatory mechanisms exist for all of the P450s. For male-specific P450g and the P450 III family, for example, pulsatile growth hormone exerts a suppressive effect, the opposite to its effect on P450h (McClellan-Green *et al.*, 1989; Waxman *et al.*, 1986).

The only clearly expressed theory of gonadal regulation of sex-related hepatic microsomal P450 expression has been that of Gustafsson, as described above. What originally consisted of a grouping of liver steroid metabolizing enzymes into at least three categories on the basis of gonadal regulation, with an emphasis on neonatal imprinting of basal levels and testosterone responsiveness, has evolved to include a role for a gonadal-hypothalamo-pituitary-liver axis in the regulation of some specific forms (Figure 1).

1.1.2.6. Unanswered Questions and Alternative Hypotheses

Many questions still remain to be addressed. The original groupings of steroid-metabolizing enzymes of Gustafsson (Einarsson *et al.*, 1973) have never been repudiated or improved, and still provide a theoretical framework (Waxman *et al.*, 1985). It is not clear how our current knowledge that multiple forms of P450 are involved in steroid

hydroxylations (Tables I & II) fits with the original groupings. The role of the gonadal-hypothalamo- pituitary-liver axis in the regulation of forms other than P450h and P450i is not known. The mechanisms involved in "imprinting" and "adult androgen responsiveness" are not known. We do not know if imprinting is necessary for both a basal level of expression and for adult androgen responsiveness for different P450 forms. And finally, and of importance to the present study, is the neonatal period the only "critical period" for imprinting?

The present study stems from recent evidence that P450 responsiveness to androgens in adult males may be "imprintable" after the neonatal period, at around the time of puberty.

Figure 1A. Gustafsson's Theory. A. Grouping of microsomal
steroid-metabolizing enzyme activities, as originally
defined (Einarsson *et al.*, 1973).

GROUP   DEFINITION

  I   enzymes irreversibly "imprinted" or "programmed"

            by androgens during the prepubertal period

            and reversibly stimulated by androgens

            postpubertally; ·

  II   enzymes with a basal activity regulated

            by nongonadal factors but reversibly inducible

            by androgens;

  III   enzymes primarily regulated by nongonadal

            factors and only slightly affected by

            androgens.

Figure 1B. Current hypothetical scheme of growth hormone regulation of certain hepatic microsomal P450 enzymes (Zaphiropoulos *et al.*, 1989).

Hypothetical scheme for GH regulation of sexually different steroid metabolism in the rat liver. In males (left) GH is released in a pulsatile fashion that induces a male type of steroid metabolism. The male type of GH secretion is regulated by hypothalamic peptides (somatostatin and GHRH) where somatostatin (produced in the anterior periventricular area) is likely to cause the troughs in male GH secretory pattern that masculinize the liver. In females (right) GH is released continuously, causing a feminized liver. The inhibition from hypothalamic centres in females is less marked, resulting in a more continuous GH release and a feminized liver. The postulated sites of action for gonadal steroids are indicated: E_2, estrogens; and T, androgens.

1.2. EVIDENCE OF IMPRINTING DURING PUBERTY OF P450 ADULT ANDROGEN RESPONSIVENESS

1.2.1. Neonatal Imprinting Is Not Always Required

1.2.1.1. Benzo[a]pyrene Hydroxylase

The only situation in which the possibility of post-neonatal imprinting has been directly tested is the hydroxylation of the carcinogen benzo[a]pyrene. Evidence has been presented by Pak and co-workers (1984) that the androgen responsiveness of the 3-hydroxylation of benzo[a]pyrene (BPH) activity can be imprinted postneonatally, at around the time of puberty. Hepatic microsomal BPH activity is approximately four-fold higher in adult males than females (Wiebel & Gelboin, 1975; Pak et al., 1984). Part of this sex-related difference is due to a responsiveness to circulating testosterone in the adult male, since castration of adult males reduces BPH activity, and testosterone treatment restores it (Kramer et al., 1979; Al-Turk et al., 1981). For BPH, adult responsiveness to testosterone appeared to require imprinting but was not neonatally imprinted, since neonatally castrated males did not respond to adult testosterone even if treated neonatally with testosterone (Pak, unpublished). This suggested a post-neonatal stage for imprinting of androgen

responsiveness of BPH activity.

Adult females appear to lack a testosterone response system for BPH, being unaffected by ovariectomy or adult testosterone administration (Pak *et al.*, 1984). However, it was possible to produce a responsiveness to testosterone for BPH in the adult female rat if testosterone treatment was given peripubertally, days 35-50 of age (Pak *et al.*, 1984), reaching approximately 40% of a normal male level of BPH activity. Further, the presence of ovaries was inhibitory to this peripubertal imprinting of androgen responsiveness by testosterone for BPH. Females gonadectomized prior to puberty, administered testosterone peripubertally, and tested for a response to androgen as adults, expressed BPH activity at a higher level than non-ovariectomized females, approximately 75% of a normal adult male.

Pubertal imprinting of testosterone responsiveness for BPH has also been demonstrated in male rats (Pak, unpublished). Male rats castrated prepubertally on day 26-28 of age, dropped to 50% of the hepatic microsomal BPH activity of the adult male, and did not respond to adult testosterone treatment unless treated with testosterone over days 35-50 of age.

While no other direct investigations of pubertal imprinting by testosterone of androgen responsiveness for BPH activity have been published, it should be realized that not all information on BPH activity is consistent with the observations of Pak and colleagues. In a study by Shimada (1987), neonatal castration reduced BPH activity, as expected. However, in contrast to the previous reports by Pak *et al.*, treatment of neonatally castrated rats with testosterone in adulthood (at 19 weeks of age) resulted in a 100% increase in microsomal BPH activity. An adult androgen response for BPH was therefore observed in male rats despite a lack of testosterone both neonatally and peripubertally. However, this discrepancy indicates the importance of using a physiological dose of testosterone to test for androgen responsiveness. In the study by Shimada *et al.* (1987), where an adult androgen response for BPH was observed, the adult testosterone dose (58 μmoles/kg of testosterone propionate on alternate days for 4-5 doses) was roughly 10-fold higher than the physiological dose used in the studies of Pak and colleagues (testosterone enanthate 2.5 μmoles/kg/day for 9 doses).

1.2.2. Which Cytochrome P450s?

In order to further investigate the possibility of post-neonatal imprinting of an androgen responsiveness, it is necessary to determine which specific hepatic microsomal P450 enzymes are involved. Based on the work of Pak *et al.* (1984; unpublished), it seems probable that one or more predominantly male P450 enzyme(s) which can catalyze the hydroxylation of BPH at the 3 position may be androgen responsive following peripubertal testosterone. At least seven different rat liver microsomal P450 forms, purified from untreated animals or animals treated with phenobarbital, PCN or β-naphthoflavone, have a demonstrated catalytic ability for BPH hydroxylation: P450s a, b, c, d, e, h (untreated), and Pb/PCN-E from the P450 III family (Guengerich *et al.*, 1982; Ryan *et al.*, 1984). P450c is the major form involved in BPH hydroxylation in 3-methylcholanthrene-induced rat liver microsomes (Ryan *et al.*, 1979), however in noninduced animals little P450c contribution is likely, since it constitutes only approximately 1% of the total microsomal P450 (Thomas *et al.*, 1981).

P450 forms which are present in higher quantities in adult male than female hepatic microsomes are P450g, P450h, RLM2 and the P450 III family (Table II). What is the evidence

that any of these are possibly androgen responsive due to peripubertal testosterone imprinting?

1.2.2.1. *P450g*

From data currently available, P450g is an unlikely candidate for pubertal imprinting of adult androgen responsiveness. P450g purified from untreated male rats does not catalyze BPH hydroxylation in a reconstituted system (Ryan *et al.*, 1984), and therefore may not be a contributor to the phenomena described by Pak *et al.* (1984) for BPH imprinting. P450g expression is male-specific, immunochemical levels of protein being detected only in liver microsomes from mature male rats, and is regulated in part by a genetic polymorphism (Ryan *et al.*, 1984; Bandiera *et al.*, 1985; Bandiera *et al.*, 1986). As well, castration at 35 days of age does not alter P450g expression in 70-day old adult male rats, therefore a dependence on adult levels of circulating testosterone is not seen (McClellan-Green *et al.*, 1989).

1.2.2.2. *P450h*

Several lines of evidence suggest that P450h might contribute to the component of BPH activity in males shown to be related to pubertally-imprintable androgen responsiveness by Pak *et al.* (1984).

P450h has been isolated by different laboratories under the names P450-male (Kamataki *et al.*, 1983), RLM5 (Cheng & Schenkman, 1982), P450-2c (Waxman & Walsh, 1983) and UT-A (Guengerich *et al.*, 1982).

Like BPH activity, hepatic microsomal P450h content (Waxman *et al.*, 1985) and testosterone 16a-hydroxylase activity (Jacobson & Kuntzman, 1969) increase dramatically in males around the time of puberty. Both P450h levels and BPH activity depend in the adult male on a response to circulating testosterone (Kamataki *et al.*, 1983; Waxman *et al.*, 1985). A correlation (r=0.850) between hepatic microsomal BPH activity and P450-male content has been demonstrated (Kato, 1987); the observed axis intercept suggests that as much as 75% of BPH activity correlates with P450-male, while approximately 25% is provided by other enzyme activity. Recently, a P450 enzyme named P450/B[a]P has been purified from untreated male rat liver microsomes (Ohgiya *et al.*, 1989). Based on immunoinhibition studies, P450/B[a]P is responsible for 80% of BPH activity in liver microsomes from untreated males. Further, P450/B[a]P is similar to P450h, based on amino-terminal sequence analysis, molecular weight and spectral properties. Hypophysectomy of adult males decreases both BPH activity (Kamataki *et al.*, 1985; Yamazoe *et al.*, 1988; Lemoine *et al.*, 1988) and P450-male content

(Kamataki *et al.*, 1985; Kato *et al.*, 1986; Shimada *et al.*, 1988).

Most importantly, full expression of P450h at normal adult male levels can be produced without neonatal exposure to testosterone. This has been observed in both male and female rats which were neonatally gonadectomized, and exposed to physiological levels of testosterone over days 35-70 of age (Dannan *et al.*, 1986). Similar results have been reported by Shimada *et al.* (1987) following testosterone administration over days 56 to 63 of age. However, it should be noted that in that study relatively high testosterone doses were used; 58 μmoles/kg/day of testosterone propionate on alternate days for 4-5 doses would have provided a dose approximately 10-fold higher than that used in the studies by Pak (1984; unpublished). These observations suggest that, as for BPH activity (Pak *et al.*, 1984), an adult responsiveness to testosterone for P450h does not depend on neonatal testosterone imprinting, and might be influenced by peripubertal testosterone. The treatment groups necessary to demonstrate a necessity for imprinting of an adult androgen responsiveness for P450h have not been included in these studies. However, it is known that neonatal testosterone treatment of neonatally castrated males restores only 40-50% of full adult expression of P450h (Kamataki *et al.*, 1983; Kamataki *et al.*, 1984; Waxman *et al.*, 1985; Dannan *et al.*, 1986;

Shimada *et al.*, 1987). Whether this deficiency involves a lack of the response system to testosterone or simply a lack of circulating testosterone in the adult is unknown.

1.2.2.3. *P450 III Family*

Our knowledge of the cytochrome P450 III family has suffered from the confusion produced by the isolation of similar yet not necessarily identical forms by many different laboratories. True to the history of P450, what was originally thought to be one enzyme has since been shown in rats to consist of two structurally similar subfamilies (Nebert *et al.*, 1987; Nebert *et al.*, 1989) (Table IV). This family is complex and not yet fully understood.

Table IV. P450III Forms in Rat Liver Microsomes. Immunochemically-related members of the P450III gene family in rats have been separated into subgroups IIIA1 and IIIA2 on the basis of amino-terminal sequences (Halpert, 1988). The forms listed under these headings are considered identical or very similar in structure and expression, however it is possible that further distinctions will be made within these groups in future. Abbreviations: UN, untreated; IND, induced with phenobarbital, PCN or trioleandomycin. References: Elshourbagy & Guzelian, 1980; Guengerich *et al.*, 1982; Waxman *et al.*, 1985; Gonzalez *et al.*, 1986; Waxman *et al.*, 1986; Halpert *et al.*, 1988; Imaoka *et al.*, 1988; Yamazoe *et al.*, 1988.

IIIA1	IIIA2	LABORATORY	ANIMAL SOURCE
PCN1	PCN2	Gonzalez	UN,IND
	Pb/PCN-E	Guengerich/	UN,IND
	Pb-2a	Waxman	
p		Guzelian	UN,IND
PCNa	PCNb,PCNc	Halpert	IND
	Pb-1	Kato	UN

The IIIA1 subfamily is induced by steroids in both sexes, however one form, PCN1, is not present at detectable levels in untreated male or female rats (Gonzalez *et al.*, 1986). Enzymes allocated by Halpert (1988) to this IIIA1 group include the pregnenolone-16α-carbonitrile (PCN)-inducible P450p (Elshourbagy & Guzelian, 1980), PCN1 (Gonzalez *et al.*, 1986), and PCNa (Graves *et al.*, 1987; Halpert *et al.*, 1988), The second subfamily has been designated IIIA2. Three IIIA2 forms, which may be identical based on amino-terminal sequences, have been named P450PCN2, which is not steroid-inducible (Gonzalez *et al.*, 1986), P450PCNb (Graves *et al.*, 1987; Halpert *et al.*, 1988), and P450 Pb-1 (Imaoka *et al.*, 1988). The laboratories of Waxman and Guengerich have reported a P450 enzyme in untreated rats which they have designated Pb-2a/PCN-E (Waxman *et al.*, 1985) or 2a (Waxman *et al.*, 1986). Halpert (1988) has reported P450 PCN-E as indistinguishable from PCNb, and thus Pb-2a/PCN-E can be tentatively allocated to the IIIA2 family, although sequencing on 2a has not been reported.

The terms IIIA1 and IIIA2 therefore do not necessarily refer to single enzymes. Nor is it clear that trivial names such as P450p necessarily refer to a single enzyme. For the purposes of this discussion, the name given by the investigators will be used in order to avoid any incorrect

assumptions.

Despite the present confusion, the following information is available based on data from untreated rats. Adult male hepatic microsomes contain higher levels of this P450 family with respect to P450 PCN2 (Gonzalez *et al.*, 1986), Pb-2a/PCN-E (Gozukara *et al.*, 1984; Waxman *et al.*, 1985; Cresteil *et al.*, 1986), and P450 Pb-1 (Imoaka *et al.*, 1988), with adult female levels being low or undetectable.

It is not clear if the developmental changes for these enzymes are parallel to those for BPH activity. In female rats, PCN2 mRNA and protein are present at birth, increase in the first week and subsequently decrease between 2 to 4 weeks of age (Gonzalez *et al.*, 1986). These results are consistent with reports for P450 Pb-1 (Imoaka *et al.*, 1988, Yamazoe *et al.*, 1988) and P450 Pb-2a/PCN-E (Waxman *et al.*, 1985), which also describe a decrease in immunochemically detectable enzyme levels around the time of puberty in the female. BPH levels in females, however, slowly increase to reach adult levels by the time of puberty (Wiebel & Gelboin, 1975; Cresteil *et al.*, 1986).

Reports on the age-related changes in P450III forms in the male have been contradictory. Both a lack of change with age

and an increase with age have been reported. Both P450PCN2 mRNA and protein increase in the first week of life in male rats, and increase further between 4 to 12 weeks of age (Gonzalez *et al.*, 1986). In contrast, P450 Pb-1 has been reported to gradually increase over days 1-30 of age and then remain high but relatively unchanging after day 30 of age (Yamazoe *et al.*, 1988). Waxman *et al.* (1985) have reported a lack of change in P450 Pb-2a/PCN-E levels over the period of of 2-12 weeks of age.

It therefore appears that some forms of P450 III increase in the male rat around the age of puberty, as seen for BPH activity (Wiebel & Gelboin, 1975; Cresteil *et al.*, 1986), while others do not. These discrepancies are based on levels of immunochemically-detectable protein and therefore may be due to differences in the specificity of the antibodies employed. It is possible that the early rise in the first week or so of life in males and females reported by Gonzalez *et al.* (1986), Yamazoe *et al.* (1988) and perhaps by Waxman *et al.* (1985) may be due to detection of an immunochemically-similar but different enzyme which is present only during early life. The detection of this enzyme may hide the rise of a second enzyme around the time of puberty which is then present as the male-specific form in the adult. Several investigators have reported a related

protein which is present early in life and then disappears (Cresteil *et al.*, 1986; Kitada *et al.*, 1987a, 1987b, 1988). Alternatively, there may be two structurally-similar P450s involved which are both expressed in the adult male, one which does not change at puberty, as described by Waxman *et al.* (1985), and one which does increase peripubertally (Gonzalez *et al.*, 1986). Hostetler *et al.* (1987) have reported two electrophoretically-separable proteins which were immunochemically-related to P450p in untreated adult males.

6β-Hydroxytestosterone is the major monohydroxylated testosterone metabolite formed by liver microsomes from immature male rats and a major metabolite in those from mature males (Lee & Park, 1989). P450III appears to be the major catalyst of testosterone 6β-hydroxylase in untreated animals, since this activity in liver microsomes is completely inhibited by antibodies against P450PCN1 (Gonzalez *et al.*, 1986) or against P450 Pb-1 (Imaoka *et al.*, 1988). It is of particular interest that testosterone 6β-hydroxylase activity in liver microsomes from untreated male rats increases peripubertally (Jacobson & Kuntzman, 1969).

It is not clear whether adult male levels of P450 III are a product of a responsiveness to circulating testosterone.

Studies of sex hormonal influences have been conducted only for P450 Pb-2a/PCN-E and 2a/PCN-E. Expression of these forms in the male shows dependence on androgen exposure, since castration at birth completely removes immunochemically detectable P450Pb-2a/PCN-E and 2a/PCN-E in the adult male rat, and neonatal testosterone partially restores the enzyme levels (Waxman *et al.*, 1985; Dannan *et al.*, 1986). Neonatal imprinting of a basal level therefore occurs. Waxman *et al.* (1985) reported that castration of males at 35 days of age did not alter immunoquantitated levels of P450 Pb-2a/PCN-E, indicating a lack of dependence on circulating androgens. In contrast, steroid 6β-hydroxylase is androgen responsive in males, being decreased by adult castration and restored by testosterone treatment (Einarsson *et al.*, 1973). In females, there is a small and variable response to adult testosterone with respect to steroid 6β-hydroxylase, suggesting a lack of androgen responsiveness (Einarsson *et al.*, 1973) as seen for BPH (Pak *et al.*, 1984).

While hypophysectomy of adult male rats decreases BPH activity (Kamataki *et al.*, 1985; Lemoine *et al.*, 1988, Yamazoe *et al.*, 1988), this surgical procedure increases P450 2a content (Waxman *et al.*, 1986).

The complexity of the P450III family and the lack of

information on different forms renders difficult any assessment of a role for hormonal regulation and imprinting. However, in neonatally gonadectomized males or females treated with physiological doses of testosterone from days 35 to 70 of age, immunochemically detectable P450 2a/PCN-E reaches the level of a normal adult male, irrespective of neonatal testosterone treatment (Dannan *et al.*, 1986). This clearly shows that neonatal testosterone is not required for complete expression of P450 2a/PCN-E. It is therefore possible, as for P450h, that testosterone exposure peripubertally, days 35-50 of age, can imprint a response to subsequent testosterone for a member of the P450III family.

1.2.2.4. P450 RLM2

Insufficient evidence is available to provide an indication of the relationship between this male-specific form and androgen exposure (Table II).

1.3. PROPOSED RESEARCH

1.3.1. Initial Focus on P450 III

From the above discussion it is clear that both P450h and P450 III forms, and possibly other P450 forms, may be influenced by peripubertal testosterone in a manner similar to BPH (Pak *et al.*, 1984). We chose to initially investigate

P450 III, since (i) the most specific P450h analysis requires either the use of specific antibodies for immunochemical quantitation, or HPLC analysis of associated testosterone hydroxylations (Waxman *et al.*, 1985; Sonderfan *et al.*, 1987). Neither of these methodologies were available at the beginning of the study, nor realistically accessible within the time limits of the study; and (ii) a relatively simple method, suitable for an initial investigative study, existed for P450 III. The demethylation of erythromycin is a catalytic activity linked to P450p (Wrighton *et al.*, 1985a; 1985b; Watkins *et al.*, 1986; Arlotto *et al.*, 1987). We decided to investigate the response of erythromycin demethylase activity to testosterone in adulthood following peripubertal testosterone exposure. The following methodology was proposed.

1.3.2. Animal Model

Female rats were chosen as the preferred animal model for the investigation of testosterone effects during a specific developmental period, since they would lack male levels of pre- and post-natal testosterone, other than that administered.

Further, prepubertal ovariectomy of these female rats was required to remove possible influences of ovarian secretions

on testosterone imprinting. Previous studies have reported an inhibition by estrogen or the presence of ovaries on imprinting by neonatal testosterone in female rats of an adult male pattern of liver cortisol metabolism (Denef & DeMoor, 1972), open field and sexual behavior (Blizard & Denef, 1973), hepatic estrogen-binding proteins (Sloop et al., 1983), hepatic demethylated epoxide metabolism (Finnen & Hassall, 1984), and growth hormone secretion pattern (Jansson & Frohman, 1987). It has been suggested that this is a mechanism by which masculinization of females by endogenous testosterone is avoided (MacLusky & Naftolin, 1981). Further, the study which has demonstrated a pubertal imprinting by testosterone of an androgen responsiveness for BPH activity clearly showed an inhibition of this imprinting in non-ovariectomized females (Pak et al., 1984). And finally, the strongest evidence for a possible peripubertal imprinting for P450h and P450III forms in females comes from a study using neonatally ovariectomized animals which therefore lacked ovaries during the putative imprinting period (Dannan et al., 1986).

As well as ovariectomized females, it was decided that male rats would be included in the study as a physiological comparison.

1.3.3. Treatment Protocol

An experimental design similar to that used by Pak *et al.*
(1984) was adopted. Thus, prepubertally ovariectomized
females were to be treated with *physiological* doses of
testosterone over a peripubertal period, defined as days
35-50 of age. A test for subsequent adult responsiveness to
testosterone would be made 30 days later. As well, a
control, non-ovariectomized group would be tested for adult
response to testosterone, indicative of the normal adult
responsiveness to androgen.

1.3.4. Specific Hypothesis

Specifically, we hypothesized that exposure to testosterone
during puberty would influence$^{\Psi}$ the responsiveness of
hepatic microsomal erythromycin demethylase activity ˏto
testosterone in adult prepubertally ovariectomized female
rats.

Corollary one: There would be a resulting increase in
erythromycin demethylase activity in females exposed to
testosterone in both periods, compared to females exposed to
testosterone during either period alone;

Corollary two: That the pattern seen in the above females
would not be identical to that in similarly treated males,
since they would have been exposed to prepubertal
testosterone and neonatally imprinted.

Ψ "influence" specifies that there must be an interaction between the two time periods of testosterone, not merely an additive effect.

2. MATERIALS AND METHODS

2.1. CHEMICALS

The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): acetyl acetone (2,4-pentanedione), ammonium acetate, erythromycin base, ethylenediaminetetraacetic acid (EDTA), semicarbazide, testosterone enanthate, and trizma base. Preservative-free corn oil was obtained from Lifestream Natural Foods, Ltd. (Richmond, B.C.). NADPH was purchased from Boehringer-Mannheim (W. Germany). Formaldehyde, magnesium chloride, potassium chloride, and sodium dithionite were obtained from BDH Chemicals Ltd. (Toronto, Canada). Sodium phosphate dibasic, sodium phosphate monobasic, and potassium phosphate monobasic were purchased from Fisher Scientific Co. (Fair Lawn, NJ). A Bradford Protein Assay Kit was obtained from Bio-Rad Laboratories (Richmond, CA). All other chemicals used were analytical reagent quality.

2.2. ANIMALS

Male and female Sprague Dawley rats were purchased from Charles River Co. (Montreal, Canada). The animals were housed in plastic-bottomed cages with corncob bedding free of antibacterials (Paxton Processing Co., Inc., Paxton, IL) under conditions of controlled temperature (23°C) and

lighting (lights on 0800 to 2200 hours). *Ad lib* access to food (Purina Rodent Laboratory Chow 5001, Ralston Purina of Canada Ltd., Woodstock, Canada) and water was provided. Gonadectomy or sham gonadectomy was performed by the breeder under ether anesthesia when the animals were 25 days of age ± one day. The animals were allowed to equilibrate for at least 8 days following surgery and 3 days following travel before treatments were started. Each treatment group initially contained four animals in the pilot study, and 9 animals in the major study.

2.3. TREATMENTS

The treatment schedules are shown in Appendix Figure 1. A solution of testosterone enanthate 4.0 mg/mL in corn oil was prepared every 5 days during the treatment periods. Peripubertal injections were given from 35 to 49 days of age. Adult testosterone treatments were administered daily from 81 to 89 days of age. In the pilot study, peripubertal injections contained either testosterone enanthate 5 μmoles/kg/day, estradiol benzoate 1.5 μmoles/kg on alternate days, or corn oil, while adult injections were either testosterone enanthate 2.5 μmoles/kg/day or corn oil. In the major study, animals received injections once daily of either testosterone enanthate 5 μmoles/kg/day or an equal volume of corn oil, in both peripubertal and adult periods.

All injections were subcutaneous and administered at the same time of day ± two hours. Animals were sacrificed at 90 days of age, at approximately 24 hours after their last injection. One animal was removed from the major study due to an error in injection.

2.4. MICROSOME PREPARATION

When the animals reached 90 days of age, they were sacrificed by decapitation. Livers were quickly removed, placed in ice-cold 0.05 M Tris, 1.15% KCl, pH 7.5, blotted, and minced. All subsequent steps were performed at 4°C. The entire liver was added to 20.0 mL of the Tris/KCl buffer, and homogenized using a Potter-Elvehjem glass mortar and a motor-driven pestle. The homogenate was centrifuged at 10,000 x g for 20 minutes. The supernatant was filtered through four layers of cheesecloth and centrifuged at 100,000 x g for 60 minutes. The resulting microsomal pellet was resuspended in 10 mM EDTA, 1.15% KCl, pH 7.4, and centrifuged again at 100,000 x g for 60 minutes. The pellet was resuspended in 4.0 mL of 0.25 M sucrose, resulting in a final microsomal protein concentration of 20-40 mg/mL. Aliquots were frozen in cryotubes at -80°C. Two animals were removed from the major study due to an error in microsome preparation.

2.5. ERYTHROMYCIN DEMETHYLASE ACTIVITY

Erythromycin demethylase activity in hepatic microsomes was determined according to the method of Arlotto *et al.* (1987), using the spectrophotometric measurement of formaldehyde formation from the method of Nash (1953). Frozen hepatic microsomes were thawed rapidly at 37°C and diluted with 0.25 M sucrose to a concentration that would give a final protein concentration in the incubation mixtures in the major study of 0.8 mg/mL (females) or 0.35 mg/mL (males). In the pilot study, final protein concentrations in the incubation mixtures were 1.1 mg/mL (females) and 0.48 mg/mL (males). The total incubation mixture contained the following components in 1.5 mL: 100 μl 6 mM erythromycin base in 30% ethanol, 100 μl 45 mM magnesium chloride, 100 μl 75 mM semicarbazide, 10 μl 150 mM NADPH in 200 mM Na,K-phosphate buffer, pH 7.4, 990 μl Na,K-phosphate buffer, and 200 μl diluted hepatic microsomes. For each sample, two blanks were assayed, one which lacked substrate during the reaction, and one which lacked microsomes. All samples were assayed in duplicate. The incubation mixtures were pre-incubated at 37°C for 75 seconds in a water bath. The reaction was started by the addition of the NADPH. After 20.0 minutes at 37°C, the reaction was stopped by the addition of 0.6 mL of ice-cold 17% perchloric acid. The tubes were vortexed and placed on ice. The components lacking in the blank tubes

were added. The tubes were centrifuged at 1000 x g for 15 muinutes at 4°C. Double Nash Reagent (ammonium acetate 7.5 g, acetyl acetone 0.1 mL, distilled water to 25 mL) was freshly prepared and 0.5 mL was added to 1.5 mL of each supernatant. These solutions were incubated at 60°C in a water bath for 15 minutes, then allowed to cool for 5 minutes. Absorbance was measured at 412 nm with a Hewlett Packard 8452A Diode Array spectrophotometer. For each sample, the net absorbance was calculated by subtracting the average absorbance of the two blanks, and formaldehyde formation was determined from a formaldehyde standard curve. Erythromycin demethylase activity was calculated as both nmoles formaldehyde/ min/ mg protein and nmoles formaldehyde/ min/ nmole P450.

2.6. PROTEIN

Hepatic microsomal protein was measured spectrophotometrically by the method of Bradford (1976). Bovine serum albumin was used as the standard. All samples were measured in duplicate. Protein content per gram wet weight of liver was estimated as (hepatic microsomal protein (mg/mL))/((liver weight(g))/(20+liver weight(mL))).

2.7. TOTAL CYTOCHROME P450

Hepatic microsomal total cytochrome P450 was measured spectrophotometrically according to the method of Omura and Sato (1964a). Hepatic microsomal samples were thawed rapidly at 37°C, and diluted 1:20 in 100 mM sodium phosphate, 20% glycerol, 0.1 mM EDTA, pH 7.4. The microsomes were reduced with a few mg sodium dithionite, and then saturated with carbon monoxide. The difference spectrum was recorded on an SLM-Aminco DW-2 spectrophotometer. Total hepatic microsomal cytochrome P450 concentration was calculated using a molar extinction coefficient of 0.091 cm^{-1} nM^{-1} (Omura & Sato, 1964b), and was expressed as nmoles P450/mg protein. Total P450 per gram wet weight of liver was estimated as (nmoles P450/mL microsome)/((liver weight(g))/(20+liver weight(mL))).

2.8. PLASMA PREPARATION

Blood was collected in test tubes containing heparin immediately following decapitation of each animal. After centrifugation at 1000 x g for 15 minutes at 4°C, the plasma was transferred into Eppendorf polypropylene micro test tubes and stored at -80°C.

2.9. PLASMA TESTOSTERONE

Total unconjugated plasma testosterone concentration was measured using a double-antibody [I^{125}] radioimmunoassay kit (ICN Biomedicals, Inc., Carson, CA). The detection limit of the assay was reported by the manufacturer as 0.1 ng/mL.

2.10. PLASMA ESTRADIOL

Total unconjugated plasma 17β-estradiol concentration was determined with a double-antibody [I^{125}] radioimmunoassay kit (ICN Biomedicals, Inc., Carson, CA). The detection limit reported by the manufacturer for this assay was 10 pg/mL.

2.11. STATISTICAL ANALYSIS

Comparisons of mean values in the treatment groups were performed using analysis of variance (ANOVA), followed by the Student Newman Keuls multiple comparison test. One-way ANOVA was used in the pilot study to examine the effect of treatments. Two-way ANOVA was used in the pilot and major studies to investigate the effects of the factors sex and treatment, or pubertal testosterone and adult testosterone, and their interactions. An interaction was indicated if the effect of one factor depended upon the presence of another factor in a non-additive manner. The correlation between hepatic microsomal erythromycin demethylase activity and plasma testosterone levels was examined using linear

regression. Differences were considered statistically significant when $p < 0.05$.

2.12. OTHER ANALYSIS

Enzyme kinetic analysis was done using the non-linear regression program Enzfitter (Leatherbarrow, 1987). Value estimates were determined from Eadie Hofstee plots. Weightings used were robust and simple, based on study of the residuals.

3. RESULTS

3.1. ERYTHROMYCIN DEMETHYLASE ASSAY CONDITIONS

The conditions used by previous investigators (Arlotto et al, 1987; Wrighton *et al.*, 1985a) for the determination of erythromycin demethylase activity in rat hepatic microsomes were verified to insure that product formation was linear with respect to both protein concentration and incubation time. Assay conditions were investigated using hepatic microsomes from both female and male untreated Sprague Dawley rats. The samples were composed of a pooled mixture of hepatic microsomes from four individual animals of the same sex. The data shown represent the results of at least one typical experiment. All experiments were repeated at least once.

3.1.1. Protein concentration

The erythromycin demethylase-related product formation in untreated male Sprague Dawley rat liver microsomes was found to be linear over the final protein concentration range in the incubation mixture of 0.11 to 0.68 mg/mL, as shown in Figure 2. In untreated female Sprague Dawley rat hepatic microsomes (Figure 3) the reaction was linear over the range 0.56 to 1.12 mg protein per mL incubation mixture. Even at this relatively high protein concentration, the product

formation indicated by absorbance from the untreated female microsomes was lower than that of the untreated male.

3.1.2. Incubation Time

Although previous investigators have used an incubation time of ten minutes (Arlotto *et al.*, 1987; Wrighton *et al.*, 1985a), the possibility of increasing the incubation time in order to increase product formation and thus absorbance was investigated. It was observed in the untreated male hepatic microsomes that, at a final protein concentration of 0.34 mg/mL, the reaction remained linear with respect to time for at least 30 minutes (Figure 4). This experiment was repeated twice at a final protein concentration of 0.34 mg/mL. The reaction was also linear over 5 to 25 minutes at a final protein concentration of 0.43 mg/mL.

In the untreated female microsomes (Figure 5) the reaction remained linear in two experiments over the first 24 minutes at a final protein concentration of 0.91 mg/mL.

3.1.3. NADPH

To insure that NADPH was not being depleted or inactivated during prolonged incubation intervals, additional NADPH equal to the amount used to initiate the reaction was added during some of the preliminary test incubations. A lack of

NADPH would be indicated by an increase in net absorbance when additional NADPH was added. As shown in Figures 6 and 7, additional NADPH midway through the incubation period decreased rather than increased the product formation, therefore depletion of NADPH was not indicated. Additional NADPH appeared to inhibit product formation.

That the assay conditions provided an excess of NADPH in the incubation mixture was further investigated in studies of erythromycin demethylase activity versus final NADPH concentration. Curvilinear plots were obtained using both untreated male (Figure 8) and untreated female (Figure 9) hepatic microsomes. In both cases, the erythromycin demethylase activity was relatively independent of NADPH at a final concentration of 1 mM. Doubling the final NADPH concentration resulted in a lower erythromycin demethylase activity, consistent with the observation made above that at high NADPH the erythromycin demethylase reaction is inhibited.

3.1.4. Substrate Concentration

The effect of varied substrate concentration on erythromycin demethylase was investigated, using final substrate concentrations ranging from 0.005 to 8 mM. At substrate concentrations below this the signal-to-noise ratio was

excessively small, while at higher substrate concentrations the relatively poor water solubility of the substrate, erythromycin base, resulted in precipitation. Direct plots of erythromycin demethylase activity versus substrate concentration are shown in Figures 10 and 11 for untreated male and female hepatic microsomes. Based on these results, a final substrate concentration of 0.4 mM was chosen for subsequent assays because (1) this concentration reflects a point on or past the shoulder of the curve, thus the substrate concentration is not limiting the rate of erythromycin demethylase activity; (2) the use of a higher substrate concentration presents the possibility of precipitation of the substrate in the incubation mixture; and (3) this is the substrate concentration used in previous reports of erythromycin demethylase activity (Arlotto *et al.*, 1987; Wrighton *et al.*, 1985a), and using the same substrate concentration allows us to compare results.

It has been reported that at least two enzymes contribute to erythromycin demethylase activity in untreated adult female Long Evans rat hepatic microsomes (Arlotto *et al.*, 1987). Representative data for untreated adult female Sprague Dawley rat hepatic microsomes are presented in Table VI and Figures 11 to 13. These experiments were performed a total of 14 times, involving from 6 to 12 points per experiment

and a substrate range of 0.005 to 4 mM. Based on the experimental data, estimated values of Michaelis-Menten constants were produced by the Enzfitter program using non-linear regression (Leatherbarrow, 1987) for both a one enzyme and a two enzyme model (Tables V-VI). The following criteria were considered:

(1) graphical analysis (Figures 11 to 13) suggested the presence of more than one enzyme; (2) the reduced chi-squared values, calculated as the weighted sum of the squared deviations of calculated from experimental values, and indicating the fit of the data (Leatherbarrow, 1987), were similar for the one component and for the two component model for the majority of data sets analyzed (Table VI);

(3) negative values for Vmax2 and Km2 were generated in the two enzyme model, but not for the one enzyme model (Table VI);

(4) for each analysis, standard errors were calculated for the estimated parameters (Table VI). These indicate the accuracy of the estimates, and for a good set of experimental data should generally not be greater than approximately 10% of the value of the parameter (Leatherbarrow, 1987). Standard errors were higher, up to 109% of the parameter estimates, in the two enzyme model, compared with a maximum 47% in the one enzyme model.

The regression analysis did not support a conclusion of

multiple enzymes, and suggested the presence of one enzyme with Vmax 0.42 nanomoles formaldehyde/min/mg protein and Km 0.04 mM catalyzing the erythromycin demethylase reaction in adult female Sprague Dawley rat hepatic microsomes. Since the graphical representations suggested multiple enzymes, a definite conclusion could not be made from the data.

Similarly, the kinetics of erythromycin demethylase in hepatic microsomes from untreated adult male Sprague Dawley rats was investigated in a total of 6 experiments, involving 6 to 12 points per run and a substrate concentration range of 0.0125 to 8 mM. Representative results are shown in Table V and Figures 10,14 and 15. It was observed that:

(1) graphical presentation suggested the presence of more than one enzyme (Figures 14 to 15);

(2) reduced chi-squared values were smaller for the one enzyme model than for the two enzyme model (Table V);

(3) negative values for Vmax2 and Km2 were generated in the two enzyme model but not in the one enzyme model (Table V);

(4) small standard errors for the parameter estimates were observed for both the one enzyme model and the two enzyme model, in some cases (Table V).

Therefore, while the most conservative interpretation of the data from untreated adult male Sprague Dawley rats indicated the presence of a single enzyme (Vmax=1.59 nanomoles

formaldehyde/min/mg protein, Km=0.12 mM) catalyzing the erythromycin demethylase reaction in hepatic microsomes, the possibility of multiple enzymes could not be discarded.

3.1.5. Phosphate Buffer

Previous investigators (Arlotto *et al.*, 1987) have specified a "potassium phosphate buffer 0.1 M, pH 7.4" for use in the erythromycin demethylase assay. The effect of different buffers on erythromycin demethylase activity is shown in Figure 16. It was observed that higher erythromycin demethylase activities were produced using a phosphate buffer composed of a mixture of 0.2 M potassium phosphate monobasic and 0.2 M sodium phosphate dibasic in a ratio of 19/81 (v/v) respectively, at pH 7.4 (Na,K-phosphate buffer 0.2 M), in comparison with either the same mixture at 0.1 M or a mixture of potassium monobasic and dibasic phosphate salts at 0.1 M.

3.1.6. Formaldehyde Standard Curve

In all assays measuring erythromycin demethylase activity performed in this study, a standard curve of formaldehyde in Na,K-phosphate buffer 0.2 M, pH 7.4, was employed to measure product formation. Since this standard curve lacked most components of the incubation mixtures, a comparison of the absorbance produced by formaldehyde in the presence and

absence of the missing components was made. Assay conditions used were those decided upon for the measurement of erythromycin demethylase activity in hepatic microsomes as described below. It was observed that the presence of hepatic microsomes, substrate, NADPH and cofactors decreased net absorbance due to formaldehyde (Figures 17 and 18). Using linear regression, slopes of net absorbance versus formaldehyde concentration were calculated and correction factors for the male assay conditions (corrected absorbance=1.18 x net absorbance) and for the female assay conditions (corrected absorbance= 1.26 x net absorbance) were determined.

3.1.7. Final conditions

Based on these preliminary studies, the final incubation mixture concentrations chosen for the measurement of erythromycin demethylase activity in rat hepatic microsomes were: microsomal protein 0.35 mg/mL (male) or 0.8 mg/mL (female), incubation time 20 minutes, erythromycin base 0.4 mM, NADPH 1 mM, and a phosphate buffer composed of a mixture of sodium phosphate dibasic 0.2 M plus potassium phosphate monobasic 0.2 M in a ratio of 81/19 (v/v) respectively (Na,K-phosphate 0.2 M), pH 7.4. The concentrations of magnesium chloride (3 mM) and semicarbazide (5 mM) were identical to those used in previous publications for the

erythromycin demethylase assay (Arlotto *et al.*, 1987; Wrighton *et al.*, 1985a).

The coefficient of variation of the assay, indicating relative error in ·separate experiments, was calculated as 14%, from eight experiments done on different days using male microsomes.

Figure 2. Erythromycin demethylase assay: absorbance (412 nm) versus protein concentration in untreated male Sprague Dawley rat hepatic microsomes (pool of 4 livers). Assay conditions: incubation time 20 minutes, final erythromycin concentration 0.4 mM, final NADPH concentration 1 mM.
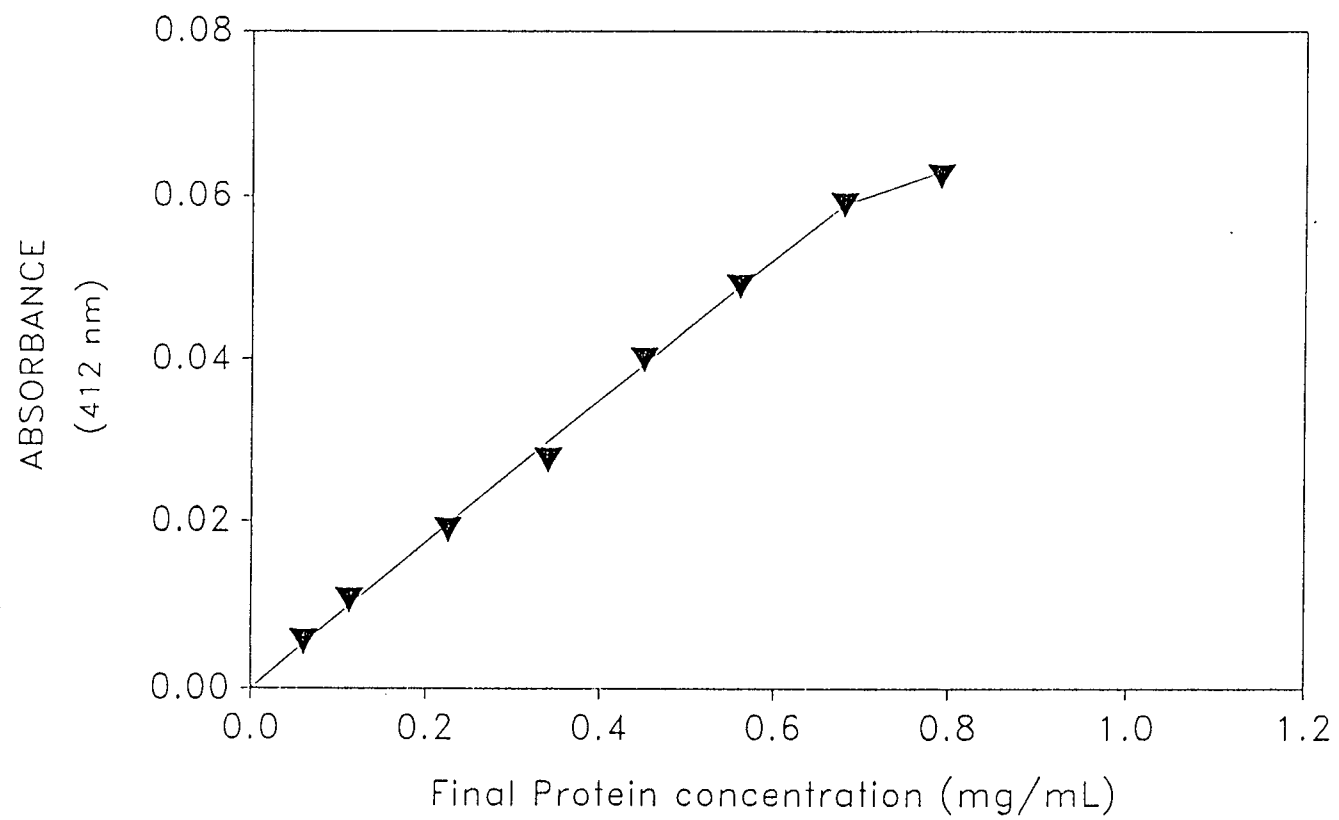
Figure 3. Erythromycin demethylase assay: absorbance (412 nm) versus final protein concentration in untreated female Sprague Dawley rat hepatic microsomes (a pool of 4 livers). Assay conditions: incubation time 20 minutes, final erythromycin concentration 0.4 mM, final NADPH concentration 1 mM.
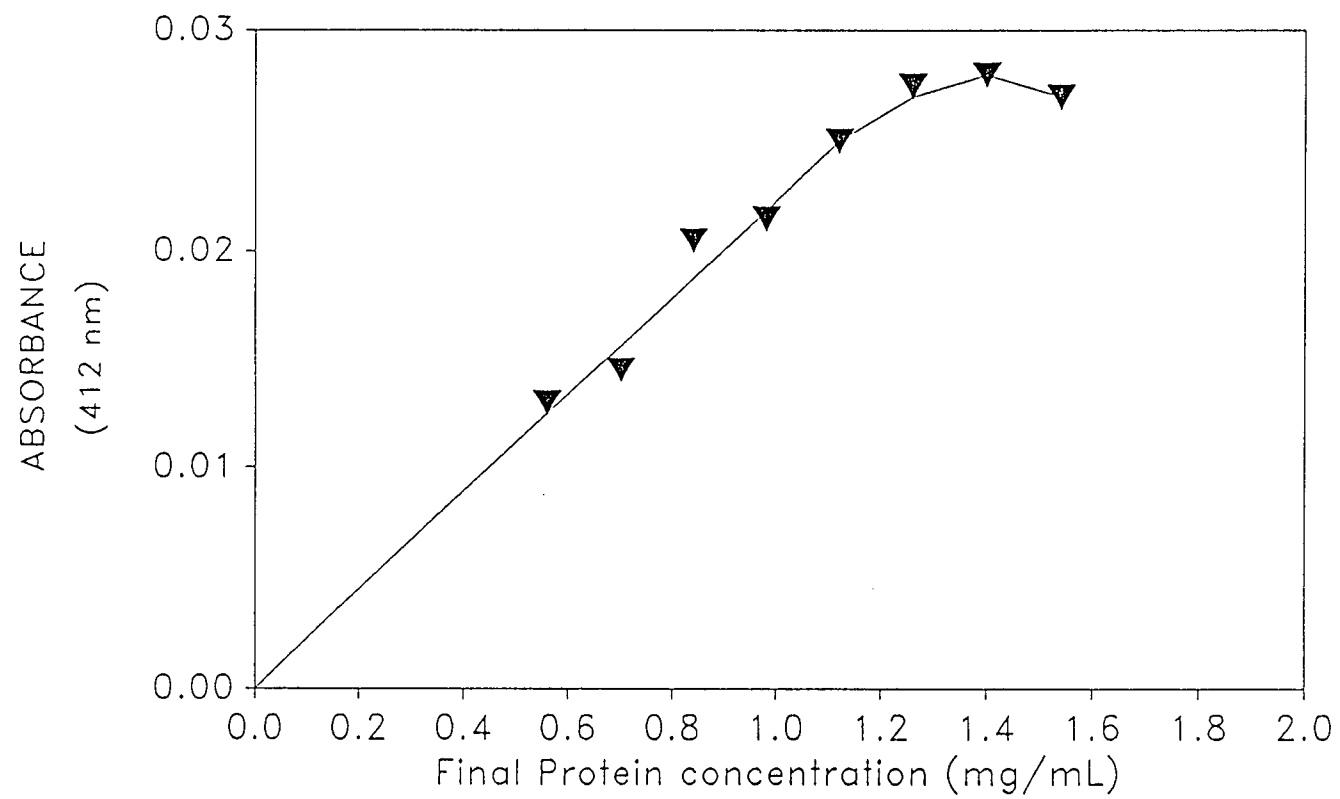
Figure 4. Erythromycin demethylase assay: absorbance (412 nm) versus incubation time in untreated male Sprague Dawley rat hepatic microsomes (a pool of 4 livers). Assay conditions: final protein concentration 0.34 mg/mL, final erythromycin concentration 0.4 mM, final NADPH concentration 1 mM.
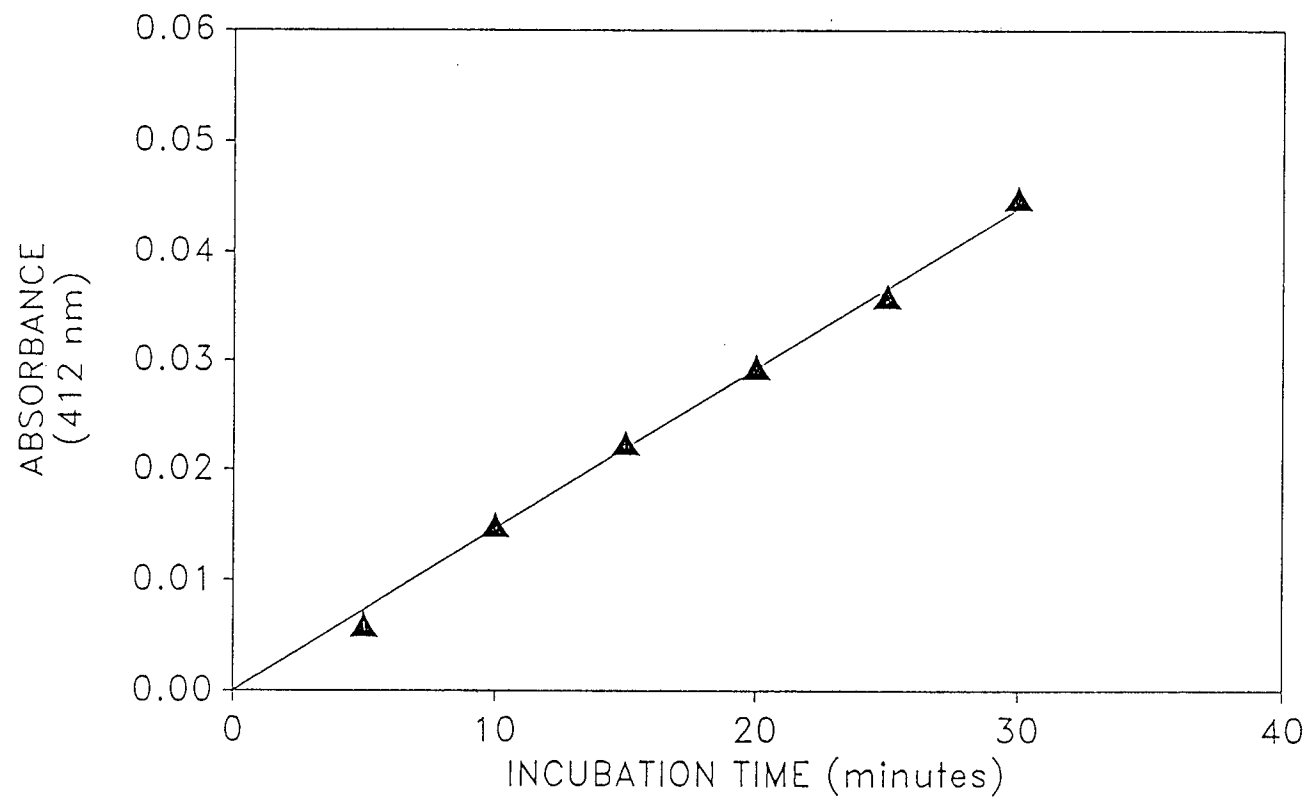
Figure 5. Erythromycin demethylase assay: absorbance (412 nm) versus incubation time in untreated female Sprague Dawley rat hepatic microsomes (a pool of 4 livers). Assay conditions: final protein concentration 0.91 mg/mL, final erythromycin concentration 0.4 mM, final NADPH concentration 1 mM.
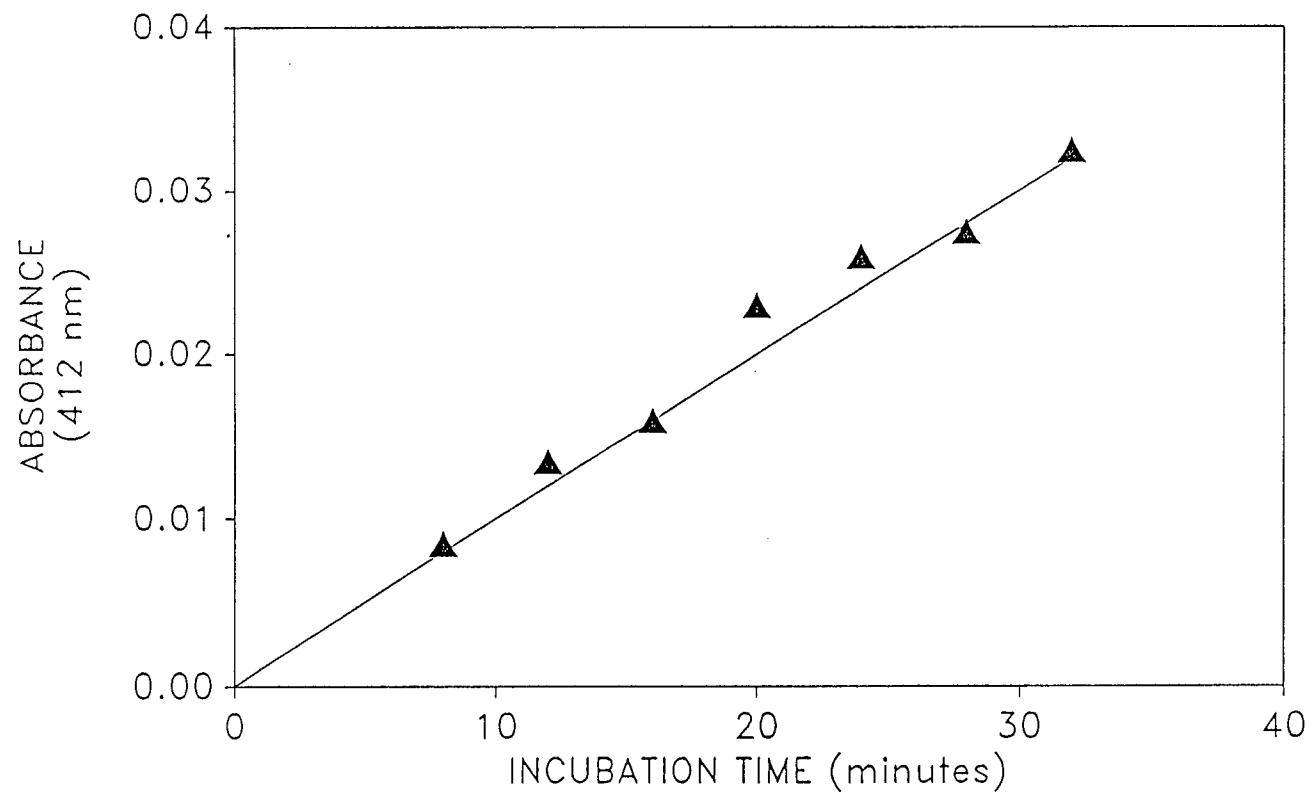
Figure 6. Erythromycin demethylase assay: absorbance (412 nm) versus incubation time, showing the effect of additional NADPH midway through the incubation period, in untreated male Sprague Dawley rat hepatic microsomes (a pool of 4 livers). Assay conditions: final protein concentration 0.43 mg/mL, final erythromycin concentration 0.4 mM, final NADPH concentration 1 mM.
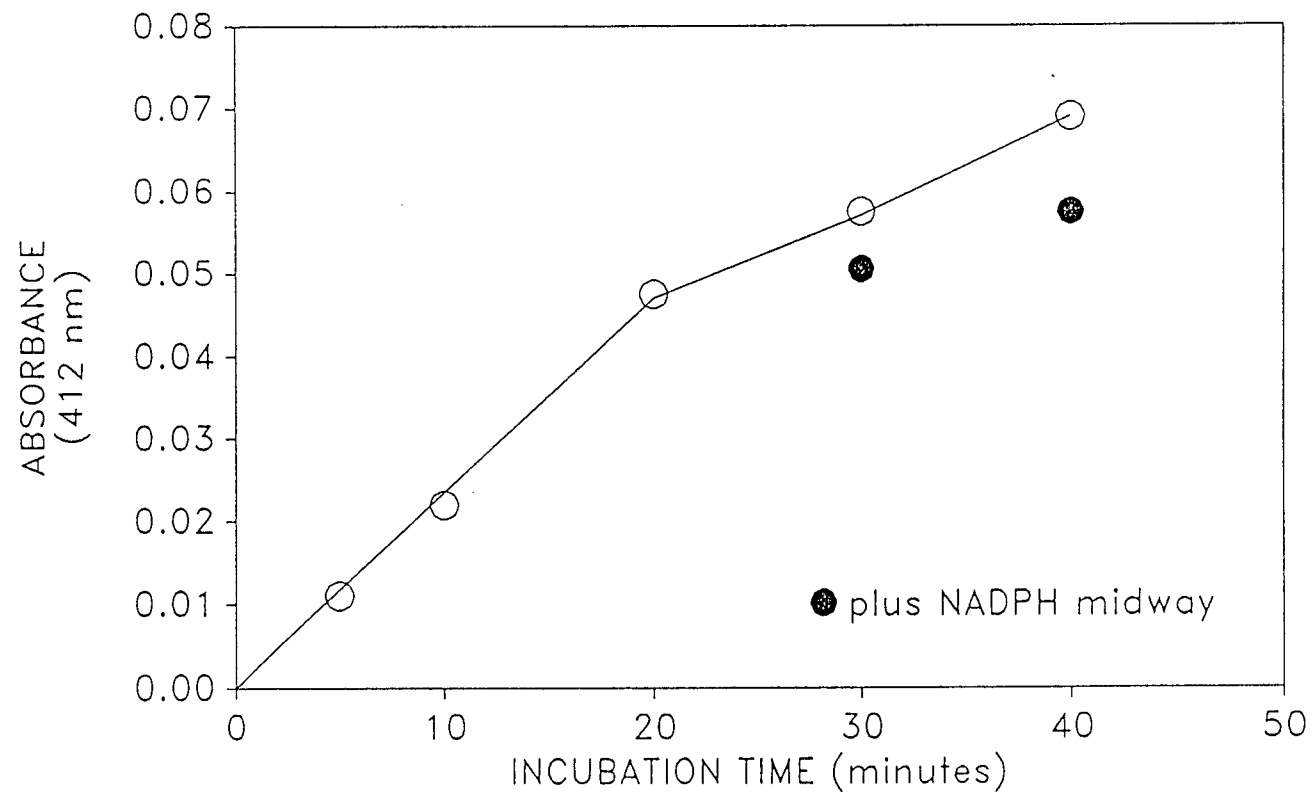
Figure 7. Erythromycin demethylase assay: absorbance (412 nm) versus incubation time, showing the effect of additional NADPH midway through the incubation period, in untreated female Sprague Dawley rat hepatic microsome (a pool of 4 livers). Assay conditions: final protein concentration 1.12 mg/mL, final erythromycin concentration 0.4 mM, final NADPH concentration 1 mM.
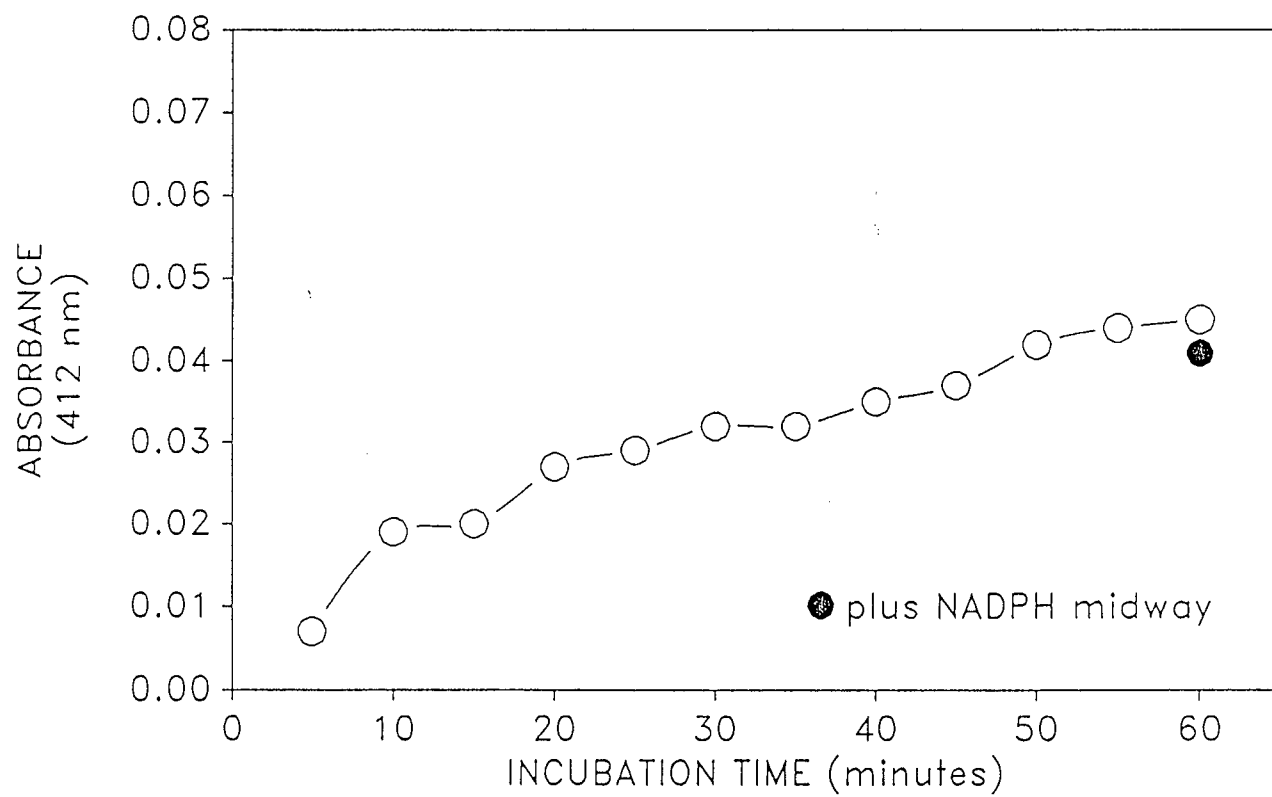
Figure 8. Erythromycin demethylase activity versus final NADPH concentration in untreated Sprague Dawley male rat hepatic microsomes (a pool of 4 livers). Assay conditions: final protein concentration 0.34 mg/mL, final erythromycin concentration 0.4 mM, incubation time 20 minutes.

ERYTHROMYCIN DEMETHYLASE
(nanomoles formaldehyde/min/mg protein)

Final NADPH concentration (mM)
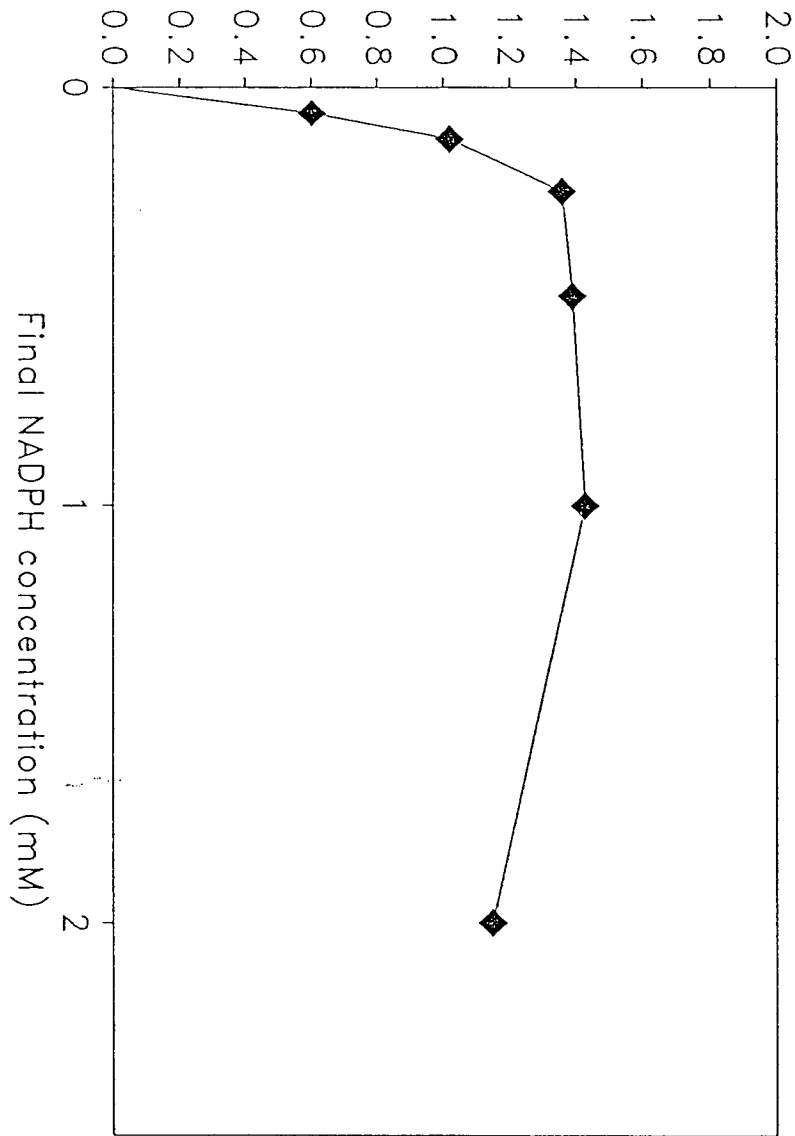
Figure 9. Erythromycin demethylase activity versus final NADPH concentration in untreated Sprague Dawley female rat hepatic microsomes (a pool of 4 livers). Assay conditions: final protein concentration 0.73 mg/mL, final erythromycin concentration 0.4 mM, incubation time 20 minutes.
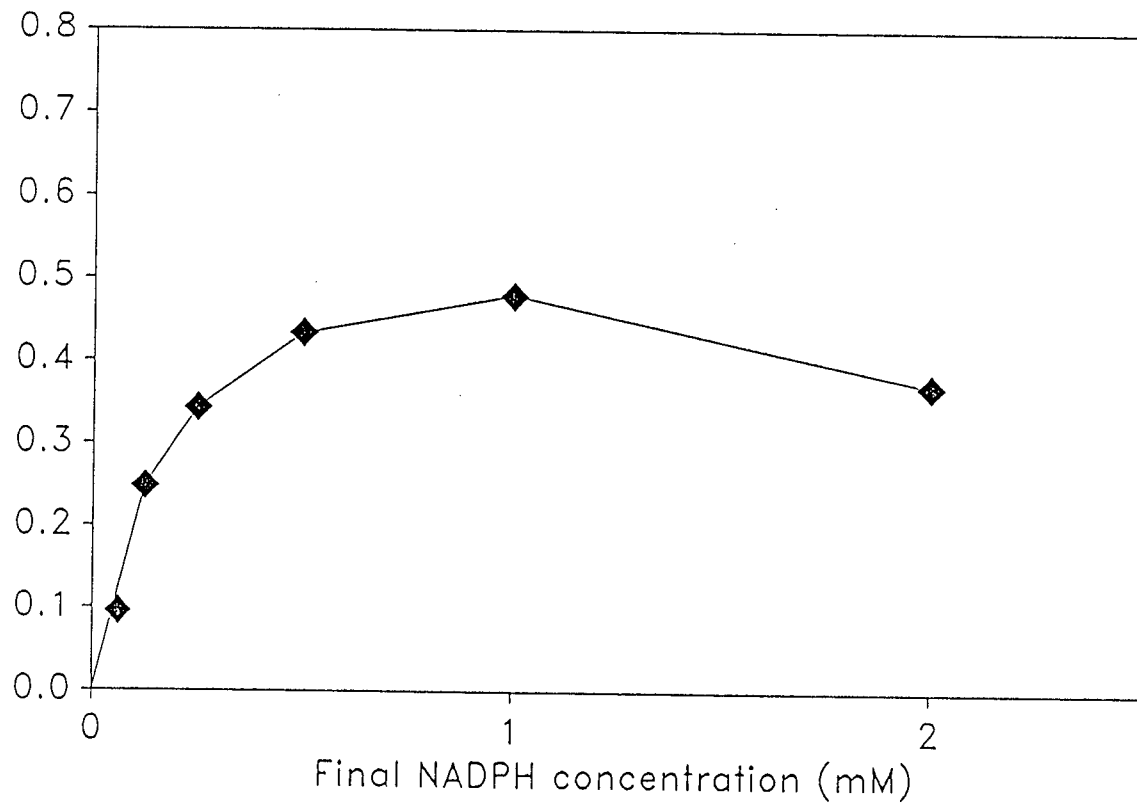
Figure 10. Erythromycin demethylase activity versus substrate concentration in untreated adult male Sprague Dawley rat hepatic microsomes (a pool of 4 livers). Assay conditions: final protein concentration 0.28-0.35 mg/mL, final NADPH concentration 1 mM, incubation time 20 minutes. Symbols indicate four separate experiments. In one of these experiments, the activity measured at 8 mM substrate did not differ from that at 1.6 mM substrate.

ERYTHROMYCIN DEMETHYLASE
(nanomoles/min/mg protein)

ERYTHROMYCIN CONCENTRATION (mM)
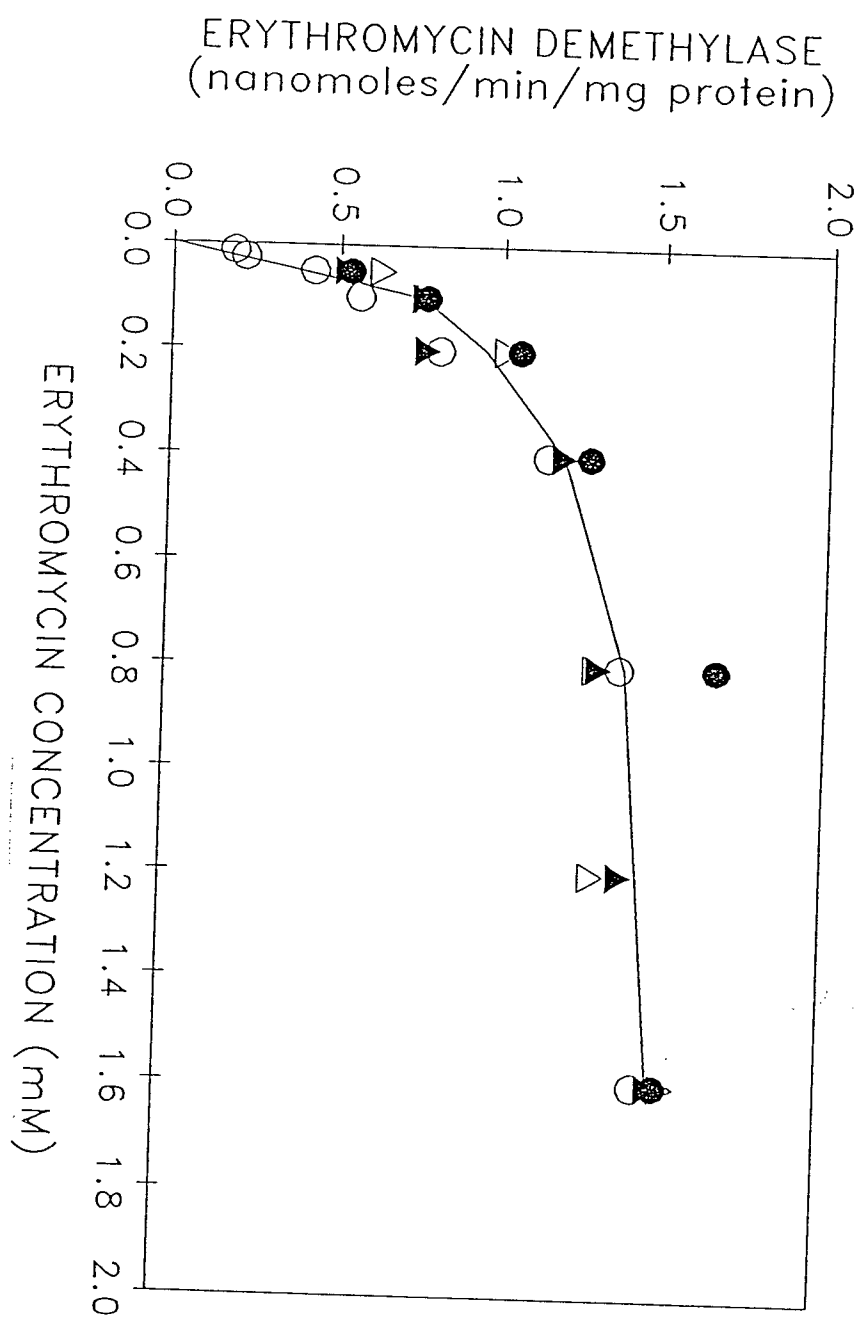
Figure 11. Erythromycin demethylase activity versus substrate concentration in untreated adult female Sprague Dawley rat hepatic microsomes (a pool of 4 livers). Assay conditions: final protein concentration 0.8-0.9 mg/mL, final NADPH concentration 1 mM, incubation time 20 minutes. Symbols indicate three separate experiments.
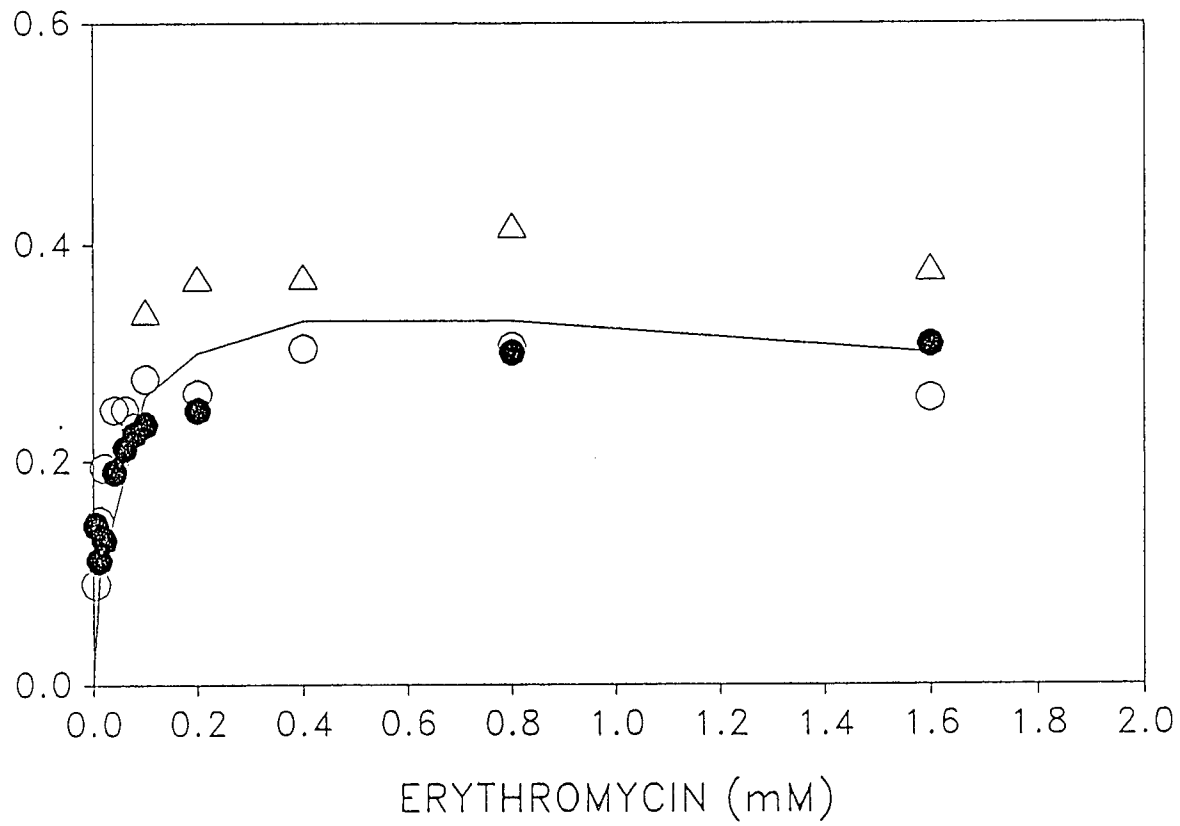
Table V. Estimated Michaelis-Menten constants in untreated male Sprague Dawley rats for erythromycin demethylase in hepatic microsomes. Data is shown as the estimated value ± S.E.M. (S.E.M. as a percentage of the estimated value).

V.

EXPT	ONE COMPONENT MODEL			TWO COMPONENT MODEL				
	Vmax	Km	Red Chi- Square	Vmax1	Km1	Vmax2	Km2	Red Chi Square
1	1.64±0.02 (1)	0.18±0.01 (6)	0.0015	3.53±0.0	6.95±0.0	-1.91±0.0	-0.0059±0.0	0.0
2	1.71±0.05 (3)	0.098±0.01 (12>	0.0036	0.69±2.9 (420)	0.075±0.52 (280)	1.02±2.86	0.12±0.44 (367)	0.0060
3	1.46±0.06 (4)	0.083±0.01 (17)	0.0049	0.71±0.001 (0.1)	0.02±0.0003 (1)	0.90±0.002 (0.2)	0.31±0.002 (0.5)	0.0056
4	1.54±0.06	0.11±0.02	0.0053	0.58±0.001	0.022±0.0004	1.12±0.0017	0.36±0.0017	0.0061
AVG	1.59±0.02	0.12±0.02		0.65±0.10	0.022±0.0			

Figure 12. Eadie Hofstee plot for erythromycin demethylase activity in untreated adult female Sprague Dawley rat hepatic microsomes. Assay conditions, as for Figure 11.
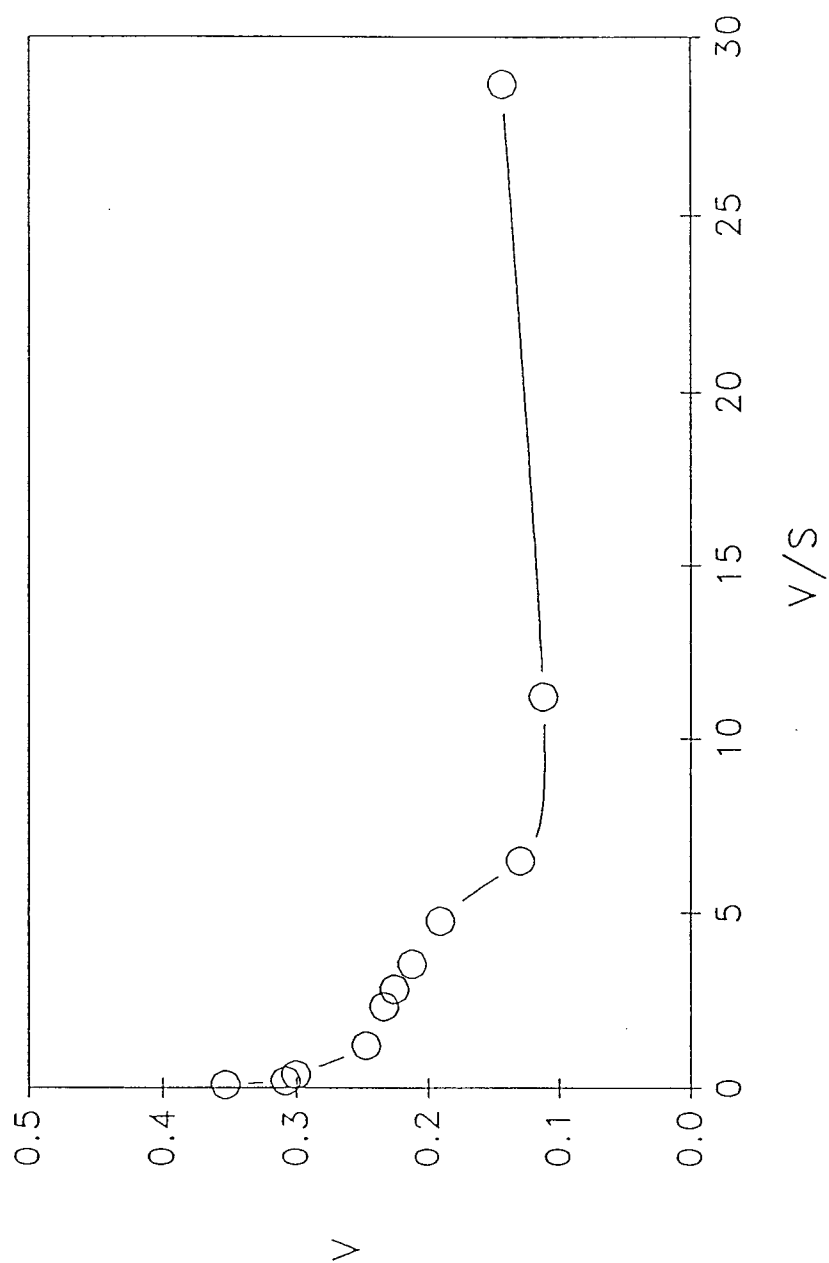
Figure 13. Lineweaver-Burk plot for erythromycin demethylase activity in untreated adult female Sprague Dawley rat hepatic microsomes. Assay conditions, as for Figure 11.
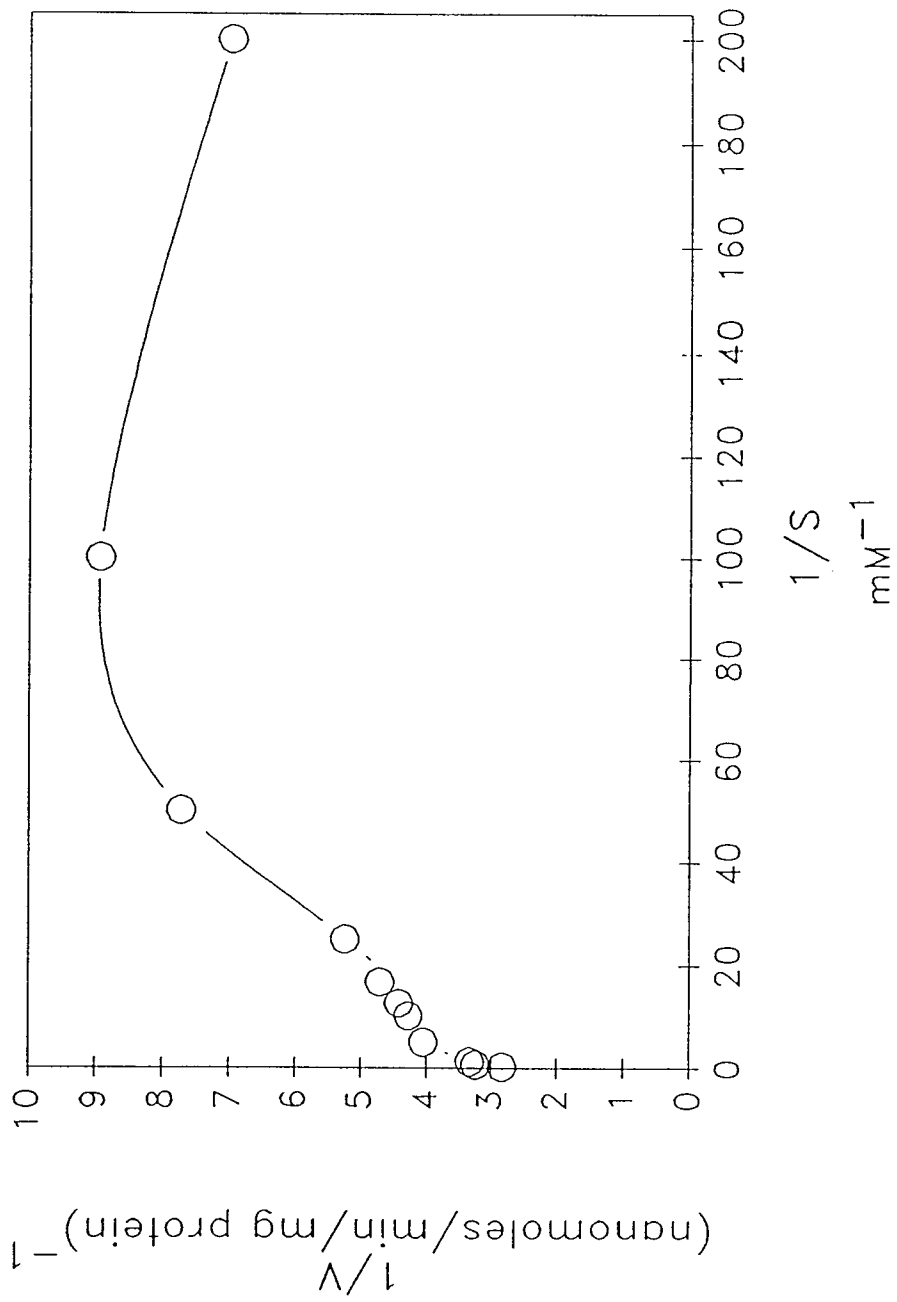
Table VI. Estimated Michaelis-Menten constants for erythromycin demethylase activity in hepatic microsomes from untreated adult female Sprague Dawley rats. Data is shown as the estimated value ± S.E.M. (S.E.M. as a percentage of the estimated value).

VI.

	ONE COMPONENT MODEL			TWO COMPONENT MODEL				
EXPT	Vmax	Km	Red Chi- Square	Vmax1	Km1	Vmax2	Km2	Red Chi Square
1	0.31±0.013 (4)	0.027±0.005 (19)	0.00052	0.30±0.03 (8)	0.040±0.009 (24)	0.02 ±0.03 (109)	-0.005 (92)	0.00029
2	0.29±0.007 (2)	0.010±0.002 (15>	0.00025	0.29±0.0002 (0.1)	0.011± 0.000047 (0.4)	-0.00056± 0.00012 (21)	-0.04±0.006 (16)	0.00029
3	0.46±0.04 (9)	0.09±0.02 (28)	0.0012	0.33±0.05 (14)	0.11±0.05 (43)	0.14±0.05 (30)	0.07±0.05 (79)	0.0015
4	0.62±0.05	0.04±0.02	0.0050	0.74±0.004	0.022±0.001	-0.11±0.003	-0.049± 0.002	0.0088
AVG	0.42±0.08	0.04±0.02		0.42±0.11	0.05±0.02			

Figure 14. Eadie Hofstee plot for erythromycin demethylase activity in hepatic microsomes from untreated adult male Sprague Dawley rats. Assay condition, as in Figure 10.
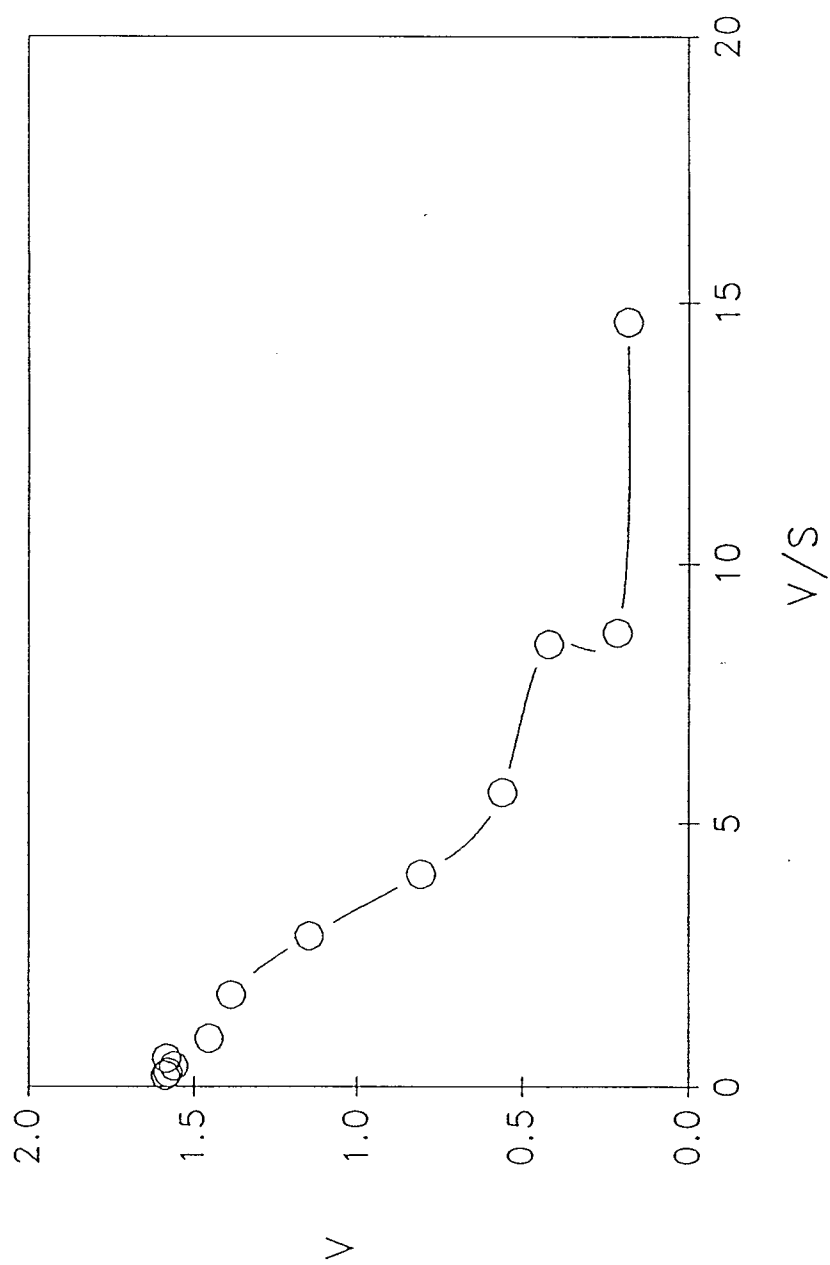
Figure 15. Lineweaver-Burk plot for erythromycin demethylase activity in hepatic microsomes from untreated adult male Sprague Dawley rats. Assay conditions, as in Figure 10.
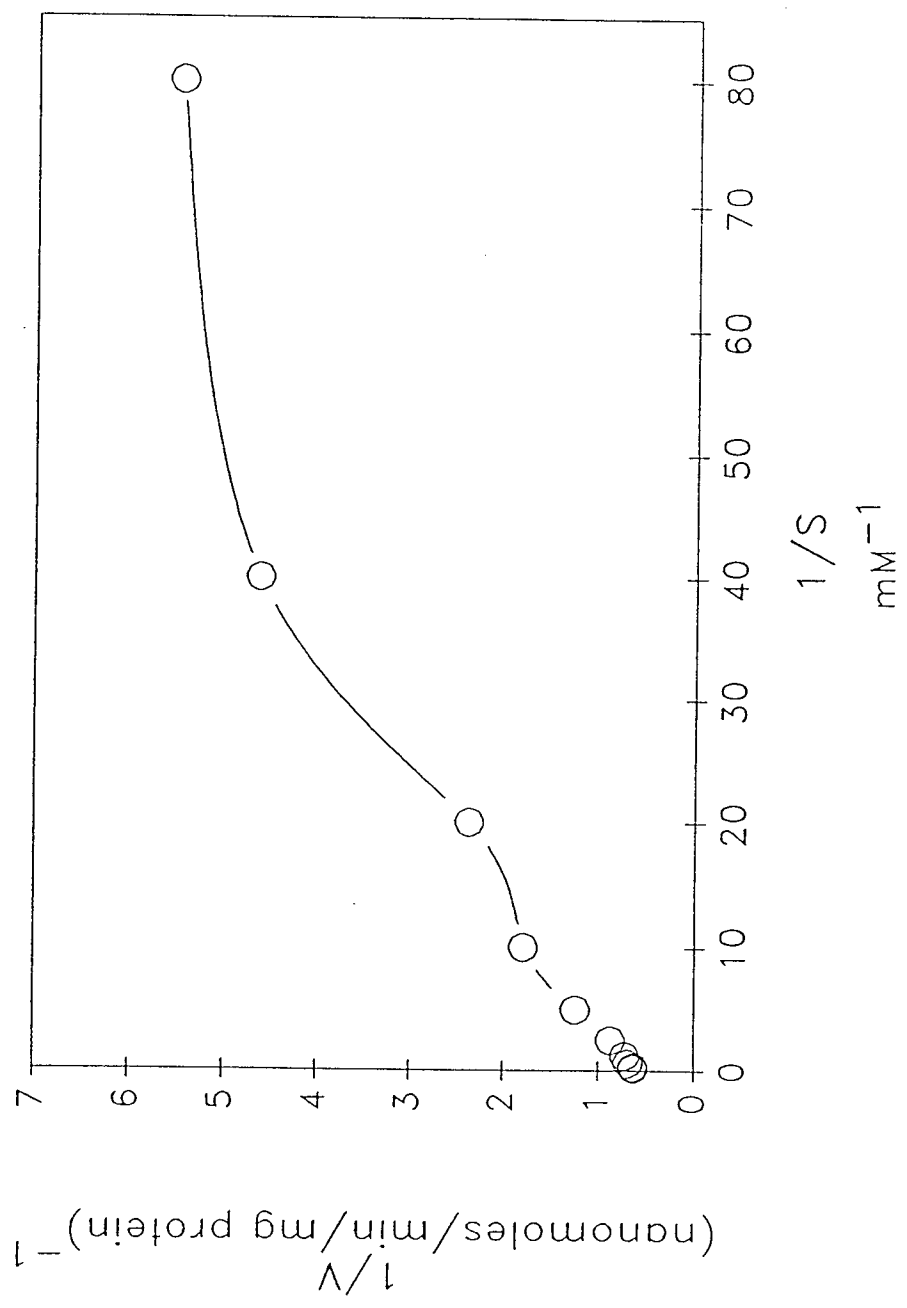
Figure 16. Erythromycin demethylase activity with varied potassium phosphate buffers in untreated male and female Sprague Dawley rat hepatic microsomes (pool of 4 livers). Assay conditions: final protein concentration 0.34 mg/mL (male) or 0.8 mg/mL (female), final erythromycin concentration 0.4 mM, final NADPH concentration 1 mM, incubation time 20 minutes.

Buffers used: Na,K-phosphate 0.2 M = potassium phosphate monobasic, sodium phosphate dibasic 0.2 M, pH 7.4;

K,K-phosphate 0.1 M= potassium phosphate monobasic and dibasic 0.1 M, pH 7.4;

Na,K-phosphate 0.1 M= potassium phosphate monobasic, sodium phosphate dibasic 0.2 M, pH 7.4.
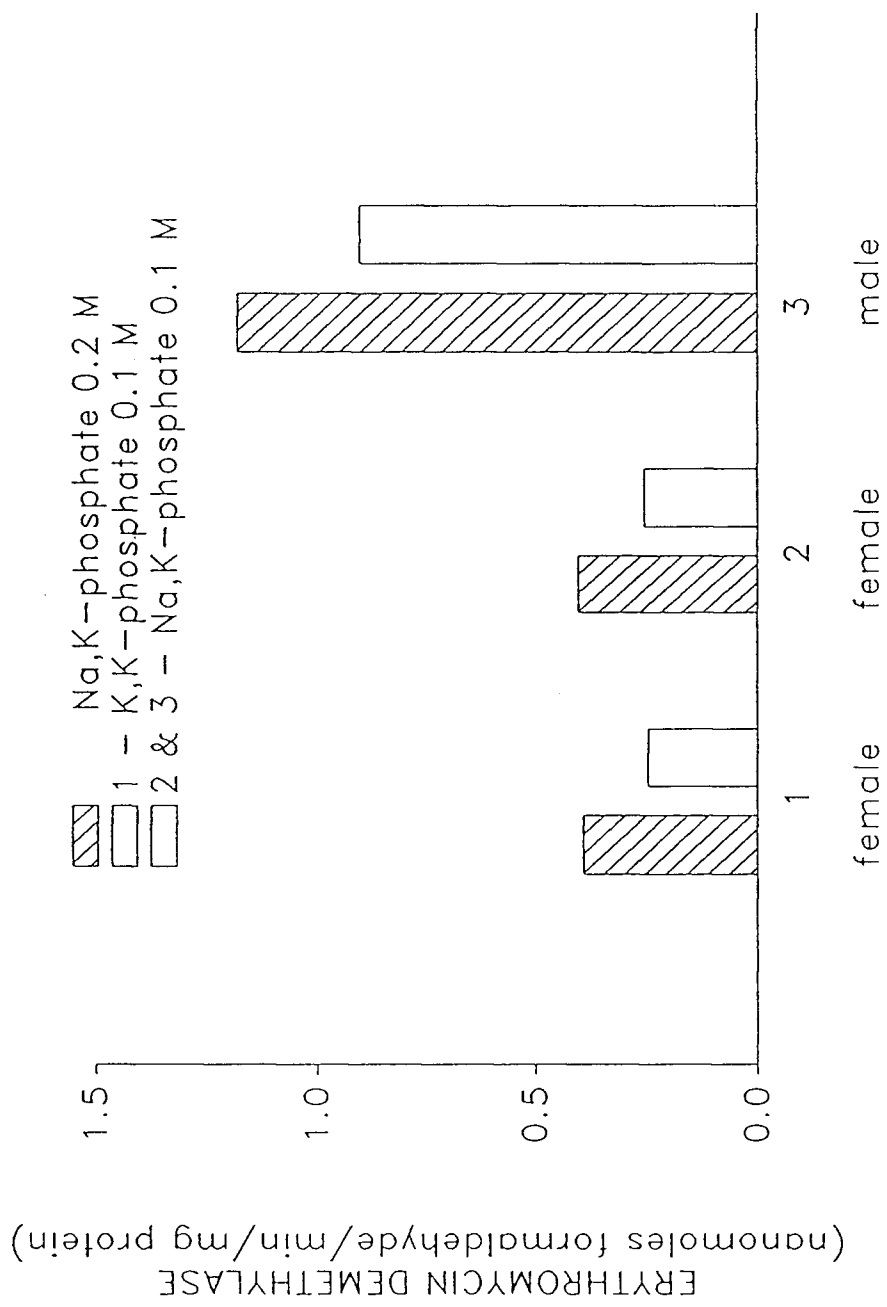
Figure 17. Formaldehyde standard curve in untreated male
Sprague Dawley rat liver microsomes (a pool of 4 livers).
Standard curves: Formaldehyde + buffer = formaldehyde in
Na,K-phosphate buffer, 0.2 M, pH 7.4; Formaldehyde + all
components = formaldehyde in a typical incubation mixture
containing untreated male hepatic microsomes (final protein
concentration 0.35 mg/mL), final erythromycin concentration
0.4 mM, final NADPH concentration 1 mM, incubation time 20
minutes, magnesium chloride 3 mM, semicarbazide 5 mM, in
Na,K-phosphate buffer 0.2 M, pH 7.4. Data shown represent
the results of linear regression analysis of duplicate
experiments.
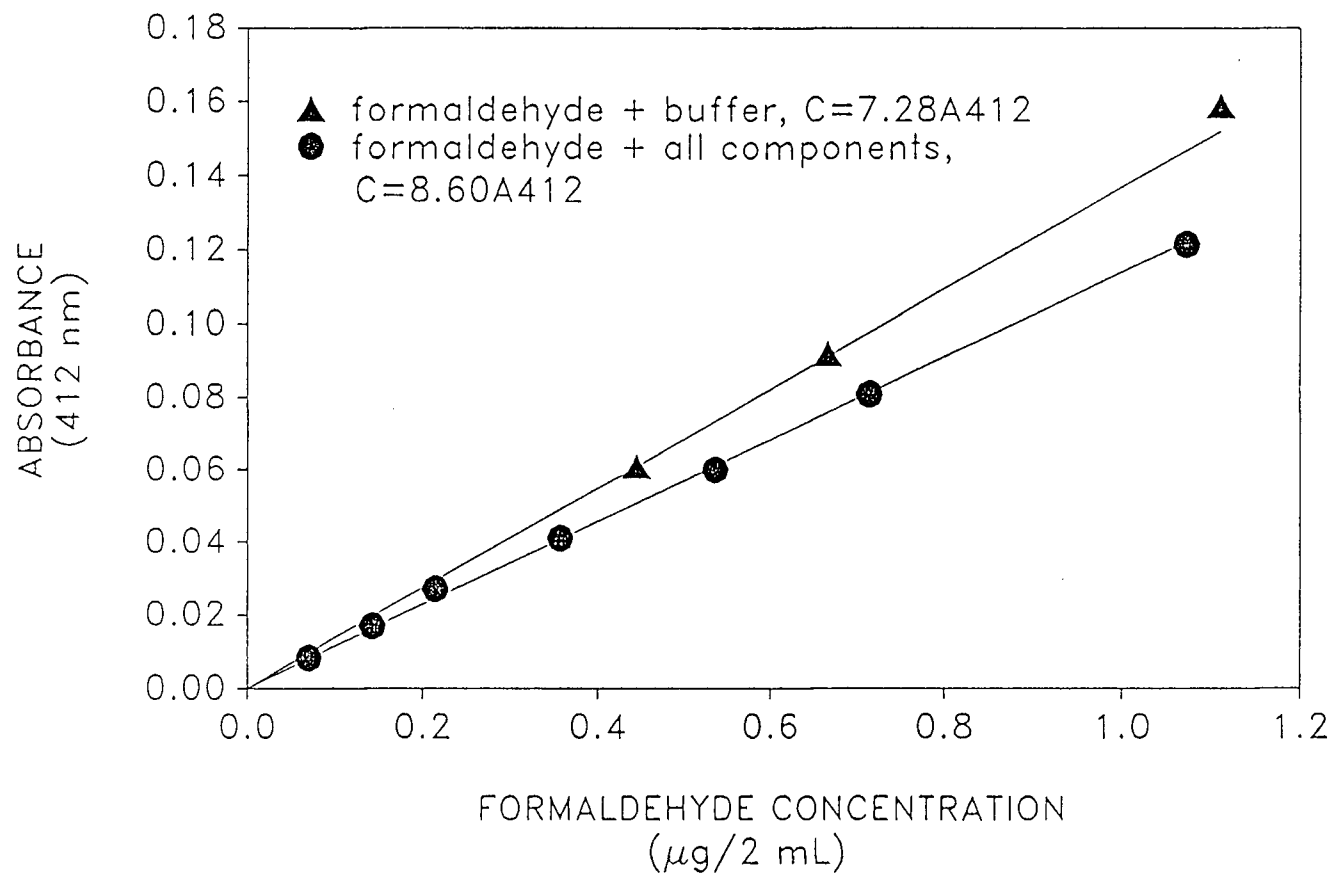
Figure 18. Formaldehyde standard curve in untreated female Sprague Dawley rat liver microsomes (a pool of 4 livers). Standard curves: Formaldehyde + buffer = formaldehyde in Na,K-phosphate buffer, 0.2 M, pH 7.4; Formaldehyde + all components = formaldehyde in a typical incubation mixture containing untreated female hepatic microsomes (final protein concentration 0.80 mg/mL), final erythromycin concentration 0.4 mM, final NADPH concentration 1 mM, incubation time 20 minutes, magnesium chloride 3 mM, semicarbazide 5 mM, in Na,K-phosphate buffer 0.2 M, pH 7.4. Data shown represent the results from linear regression analysis of duplicate experiments.
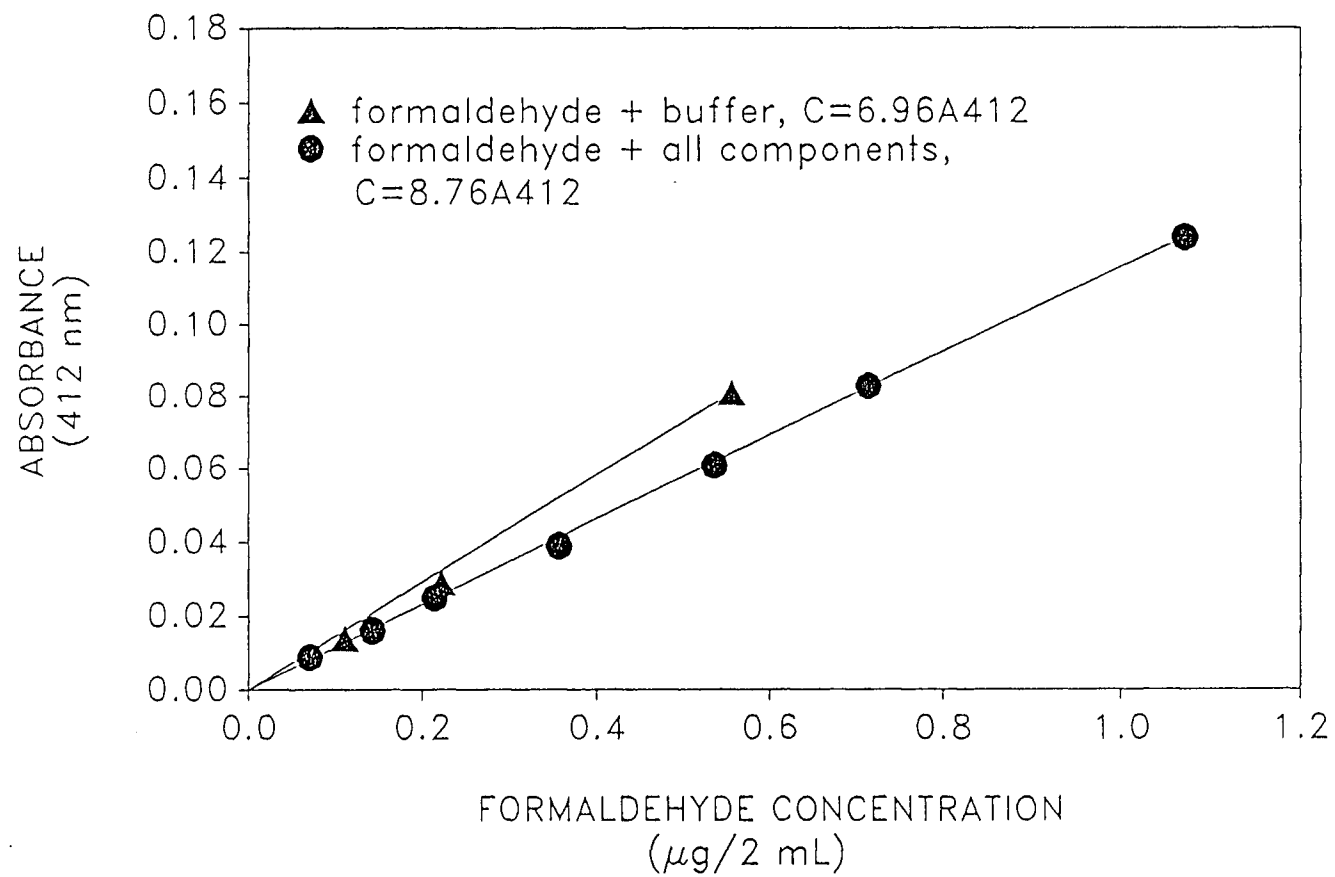
3.2. ERYTHROMYCIN DEMETHYLASE STABILITY

Since erythromycin demethylase activity would be assayed in hepatic microsomes which had been stored at $-80°C$ for several months, the stability of this enzyme activity over time was investigated. Hepatic microsomes from untreated adult female Sprague Dawley rats were assayed for erythromycin demethylase activity immediately after preparation, then stored at $-80°C$. As shown in Figure 19, the erythromycin demethylase activity in aliquots sampled after being frozen, and then thawed at various time points, was unchanged for up to 300 days (10 months).

Figure 19. Erythromycin demethylase activity: stability in adult female Sprague Dawley rat hepatic microsomes stored at -80°C (a pool of 4 livers). Assay conditions: final protein concentration 0.86 mg/mL, erythromycin 0.4 mM, NADPH 1mM, incubation time 20 minutes. __, calculated average; ---, ± one standard deviation from the average.

ERYTHROMYCIN DEMETHYLASE
(nanomoles formaldehyde/min/mg protein)

TIME FROZEN AT −80 C
(days)
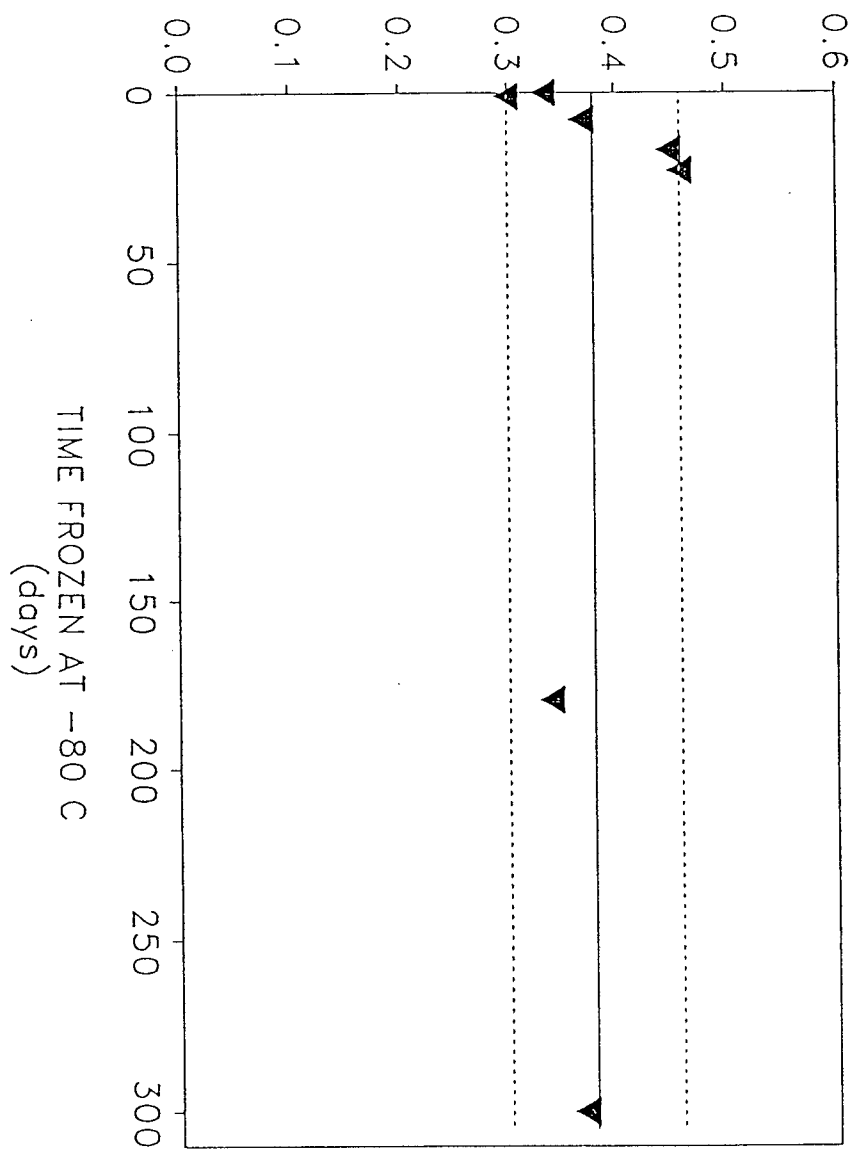
3.3. THE PILOT STUDY

3.3.1. Pubertal Testosterone Treatment in Ovariectomized Females

Since the research question was primarily concerned with the effect of pubertal testosterone on the adult androgen responsiveness of hepatic microsomal erythromycin demethylase activity in prepubertally ovariectomized female rats, the data from only the four groups specifically related to this question were examined separately (Table VII, Appendix Table A, Figures 20 and 21). As well, other comparisons were of interest when all study groups were considered (Table VIII, Appendix Table B, Figures 20 and 21).

3.3.1.1. Hepatic Microsomal Erythromycin Demethylase Activity

Hepatic microsomal erythromycin demethylase activity was calculated on the basis of both microsomal protein and microsomal P450 concentration. The results are presented in Table VII and Figures 20 and 21. A two-way ANOVA (2x2) was performed (Appendix Figure 2), with pubertal testosterone and adult testosterone as the two factors. A statistically significant interaction between these two factors was not detected (p=0.091 (protein), p=0.058 (P450), however each factor alone had a significant effect (p=0.012 and 0.024 (protein), p=0.015 and 0.014 (P450). By multiple comparison

test, erythromycin demethylase activity was significantly higher in prepubertally ovariectomized female rats which were exposed to testosterone both peripubertally and in adulthood, when compared to ovariectomized females treated with testosterone during either period alone.

3.3.1.2. Cytochrome P450 and Protein

The effects of peripubertal and adult testosterone on the content of P450 and of protein in the liver were examined for the four prepubertally ovariectomized female groups, as shown in Appendix Table A. Neither peripubertal testosterone, adult testosterone nor the combination of the two treatments significantly altered microsomal P450 (nmoles P450/mg protein), the hepatic P450 (P450/g wet weight of liver) or the hepatic protein content (mg protein/g wet weight of liver) in these groups.

3.3.2. All Treatment Groups

3.3.2.1. Hepatic Microsomal Erythromycin Demethylase Activity

Erythromycin demethylase activity was measured in individual hepatic microsomal samples from study animals in all treatment groups. These results are presented based on microsomal protein concentration as well as based on cytochrome P450 concentration (Table VIII, Figures 22 and

23). By either method of calculation, mean hepatic microsomal erythromycin demethylase activity was higher, approximately 2 - fold, in control males than in control females. Oneway ANOVA indicated a significant difference between female treatment groups for both calculations. By either calculation, ovariectomy on day 25 of age resulted in a 38% decrease in mean hepatic microsomal erythromycin demethylase activity from control female levels, however this decrease was not statistically significant, either by ANOVA or by Student's T-test. Neither estradiol nor testosterone administration in any single time period significantly altered mean erythromycin demethylase activity compared with control females.

The combination of peripubertal testosterone and adult testosterone treatments in ovariectomized females significantly increased erythromycin demethylase activity compared to ovariectomized females not given testosterone by 103% (by protein) and by 106% (by P450). The hepatic microsomal erythromycin demethylase activity in ovariectomized females given both peripubertal and adult testosterone did not differ statistically from that of control females. The hepatic microsomal erythromycin demethylase activity in prepubertally ovariectomized females given both testosterone treatments reached 57% (by protein)

and 68% (by P450) of average control male activity.

The mean hepatic microsomal erythromycin demethylase activity in the group treated with peripubertal estradiol and adult testosterone did not differ statistically from that of the ovariectomized female group.

3.3.2.2. *Liver Cytochrome P450 and Liver Protein*

Both protein and P450 concentrations in the livers from study animals were assessed. These results were analysed by one-way ANOVA. As shown in Appendix Table B, no significant differences were found between the different study groups with respect to hepatic microsomal P450 content (nmoles cytochrome P450/mg microsomal protein). Similarly, the amount of P-450 or protein per gram wet weight of liver did not differ between the treatment groups.

3.3.2.3. *Body and Liver Weights*

The mean body and liver weights at the conclusion of the study are shown in Appendix Tables B and C. One-way ANOVA and multiple comparison tests revealed many differences between the female treatment groups for these parameters. Of interest was the observation that the average body weight and liver weight in the prepubertally ovariectomized female group treated with both pubertal and adult testosterone were

significantly higher than observed in the control female group, although the average liver weight as a percentage of total body weight was not different.

Table VII. Pilot study: the influence of pubertal testosterone on ovariectomized female rat liver hepatic microsomal erythromycin demethylase activity. Data is presented as mean ±SEM (n=4), and is also included in Table VIII. Abbreviations: EDM, hepatic microsomal erythromycin demethylase activity; per protein, nmoles formaldehyde/min/mg protein; per P450, nmoles formaldehyde/min/nmole P450; Fem, female; Gx, prepubertally ovariectomized; Ta, adult testosterone treatment; Tp, peripubertal testosterone treatment; "*" indicates a significant difference from all other groups in that row (p<0.05, two-way ANOVA).

	TREATMENT GROUP			
	Fem	Fem	Fem	Fem
	Gx	GxTa	GxTp	GxTpTa
EDM				*
per protein	0.29±0.05	0.33±0.04	0.35±0.04	0.59±0.08
EDM				*
per P450	0.36±0.08	0.41±0.04	0.41±0.04	0.74±0.10

Figure 20. Pilot study: erythromycin demethylase calculated on the basis of microsomal protein in ovariectomized female rats (mean ± S.E.M., n=4). These results were also included in Figure 22. "*" indicates a significant difference from all other treatment groups ($p < 0.05$). Animals received the following treatments:

Gx Fem: gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

GxTa Fem: gonadectomy day 25, corn oil days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

GxTp Fem: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, corn oil days 81-90.

GxTpTa Fem: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

ERYTHROMYCIN DEMETHYLASE
(nanomoles formaldehyde/min/mg protein)
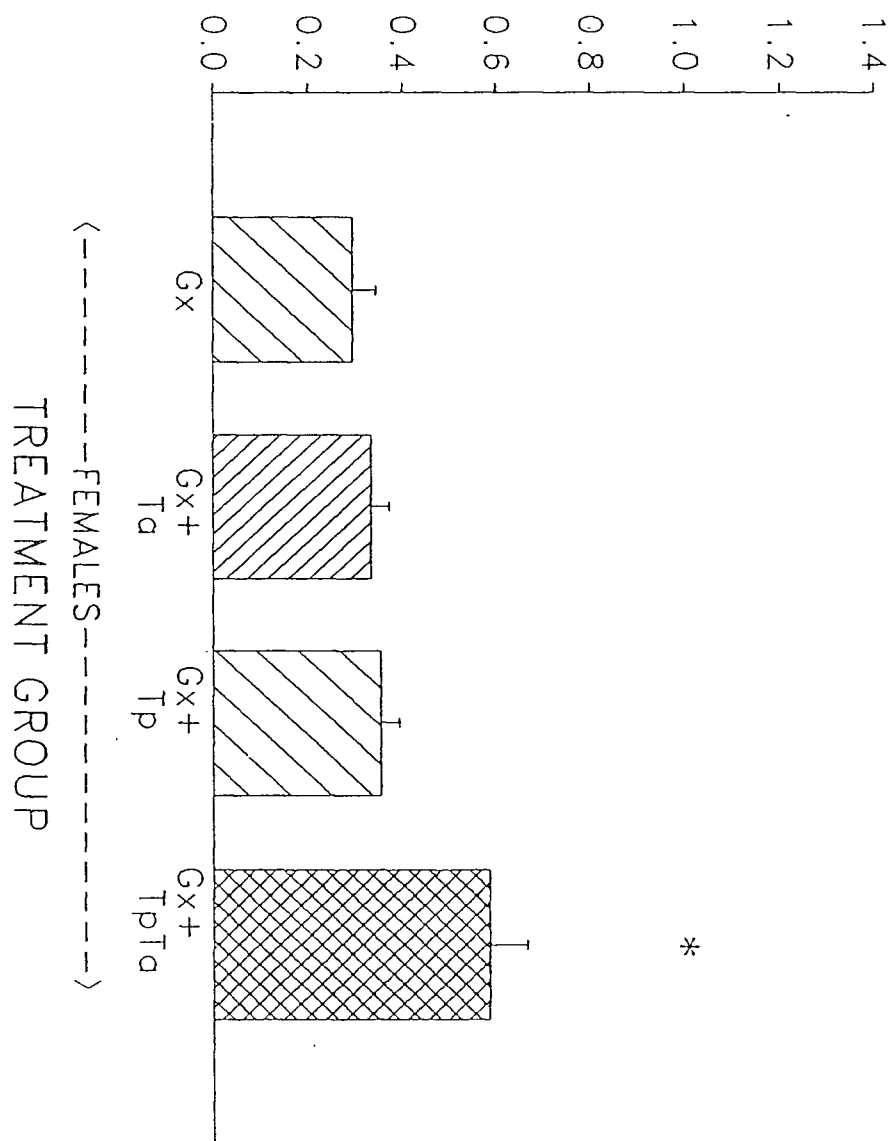
Figure 21. Pilot study: erythromycin demethylase calculated on the basis of cytochrome P450 content in ovariectomized females. mean ±S.E.M., n=4) "*" indicates a significant difference from all other treatment groups ($p < 0.05$). These results were also included in Figure 23. Animals received the following treatments:

Gx Fem: gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

GxTa Fem: gonadectomy day 25, corn oil days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

GxTp Fem: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, corn oil days 81-90.

GxTpTa Fem: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

ERYTHROMYCIN DEMETHYLASE
(nanomoles formaldehyde/min/nmole cytochrome P−450)
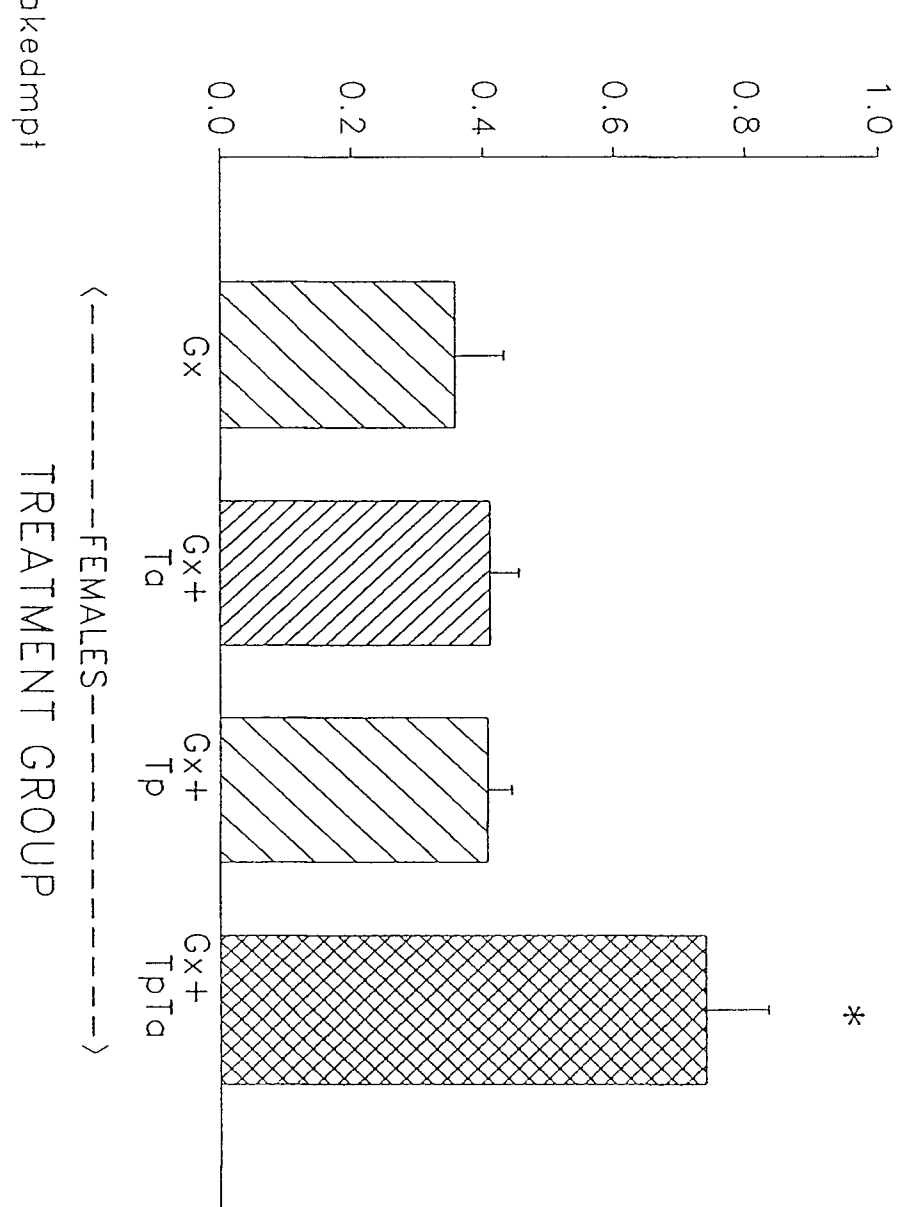
Table VIII. Pilot study: hepatic microsomal erythromycin demethylase activity in all treatment groups. Data is presented as mean ±S.E.M. (n=4). Abbreviations: EDM, erythromycin demethylase; per protein, nmoles formaldehyde/min/mg protein; per P450, nmoles formaldehyde/min/nmole P450; Fem, female; Gx, prepubertal ovariectomy; Ep, pubertal estradiol; Ta, adult testosterone; Tp, peripubertal testosterone. "*" indicates a significant difference from the mean for the Fem Gx, FemGxTa, FemGxTp and FemGxEp groups in that row (one-way ANOVA, female groups, $p < 0.05$).

Table
VIII.

	CON MALE	CON FEM	FEM Gx	FEM GxTa	FEM GxTp	FEM GxTpTa	FEM GxEp	FEM GxEpTa
EDM per protein	1.03±0.13	0.47±0.07	0.29±0.05	0.33±0.04	0.35±0.04	* 0.59±0.08	0.36±0.04	0.46±0.05
EDM per P450	1.09±0.16	0.58±0.10	0.36±0.08	0.41±0.04	0.41±0.04	* 0.74±0.10	0.42±0.03	0.57±0.04

Figure 22. Pilot study: Hepatic microsomal erythromycin demethylase calculated on the basis of microsomal protein in all groups. (mean ±S.E.M., n=4). "*" indicates a significant difference from the mean for groups FemGx, FemGxTa, FemGxTp, and FemGxEp (one-way ANOVA, female groups, $p<0.05$). Animals received the following treatments:

Con Male: sham gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

Con Fem: sham gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

Gx Fem: gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

GxTa Fem: gonadectomy day 25, corn oil days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

GxTp Fem: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, corn oil days 81-90.

GxTpTa Fem: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

GxEp Fem: gonadectomy day 25, estradiol benzoate in corn oil 1.5 μmoles/kg on alternate days, days 35-50, corn oil days 81-90.

GxEpTa Fem: gonadectomy day 25, estradiol benzoate in corn oil 1.5 μmoles/kg on alternate days, days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

ERYTHROMYCIN DEMETHYLASE
(nanomoles formaldehyde/min/mg protein)

Figure 23. Pilot study: hepatic microsomal erythromycin demethylase activity calculated on the basis of P450 content in all groups. Values are expressed as mean ±S.E.M. (n=4). "*" indicates a significant difference from the means for groups FemGx, FemGxTa, FemGxTp and FemGxEp (one-way ANOVA, female groups, $p < 0.05$). Animals received the following treatments:

Con Male: sham gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

Con Fem: sham gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

Gx Fem: gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

GxTa Fem: gonadectomy day 25, corn oil days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

GxTp Fem: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, corn oil days 81-90.

GxTpTa Fem: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

GxEp Fem: gonadectomy day 25, estradiol benzoate in corn oil 1.5 μmoles/kg on alternate days, days 35-50, corn oil days 81-90.

GxEpTa Fem: gonadectomy day 25, estradiol benzoate in corn oil 1.5 μmoles/kg on alternate days, days 35-50, testosterone enanthate in corn oil 2.5 μmoles/kg/day days 81-90.

ERYTHROMYCIN DEMETHYLASE
(nanomoles formaldehyde/min/nmole cytochrome P-450)

3.4. THE MAJOR STUDY

3.4.1. Prepubertally Ovariectomized Female Rats

Since the research question primarily concerned the influence of peripubertal testosterone on adult androgen responsiveness in prepubertally ovariectomized females, only those four groups specifically addressing this question were considered first.

3.4.1.1. Hepatic Microsomal Erythromycin Demethylase Activity

Erythromycin demethylase activity was calculated on the basis of both microsomal protein and microsomal P450 in the incubation mixture. The results are shown in Table IX and Figures 24 and 25. A two-way ANOVA (2 by 2, pubertal testosterone by adult testosterone, Appendix Figure 3) indicated a significant interaction (p=0.003, power=0.99) between pubertal testosterone and the response to adult testosterone in erythromycin demethylase activity in ovariectomized females when activity was calculated on the basis of protein. A significant interaction was not indicated (p=0.217, power<0.3) when erythromycin demethylase activity was calculated on the basis of microsomal P450. By either calculation, both pubertal testosterone and adult testosterone significantly (p=0.000) affected erythromycin demethylase activity, and multiple comparison testing

indicated that the combination of both treatments resulted in a significantly higher erythromycin demethylase activity compared with the other three groups (Figures 24 and 25). Erythromycin demethylase activity was 77% higher (by either calculation) in ovariectomized females which received both adult and peripubertal testosterone than in ovariectomized females which were not given testosterone. The pattern of erythromycin demethylase activity within these four groups was similar to that seen in the pilot study (Figure 26).

3.4.1.2. Cytochrome P450 and Protein

Since hepatic microsomal erythromycin demethylase activity results differed slightly depending on the method of calculation used, the effects of peripubertal and adult testosterone on hepatic microsomal P450 and on liver P450 and protein content in the four peripubertally-ovariectomized groups were examined. As shown in Table IX, the protein content of the liver was unaffected by testosterone treatment in ovariectomized females. Testosterone treatments did have an effect on the P450 content of the liver. By two-way ANOVA (2 by 2, adult testosterone and peripubertal testosterone), a significant interaction was indicated. The combined pubertal and adult testosterone treatment resulted in a liver P450 content which was significantly higher than that following either

testosterone treatment alone. Hepatic microsomal P450 content (nmoles P450/mg protein, Table IX) reflected the liver P450 concentrations, however no significant differences were detected. These results will therefore affect the calculation of hepatic microsomal erythromycin demethylase activity per nmole P450.

3.4.1.3. Body and Liver Weights

Body and liver weights in the four ovariectomized female rat groups at the conclusion of the study are shown in Table IX. By two-way ANOVA, adult testosterone significantly increased both body weight and liver weight in the prepubertally ovariectomized female animals. When liver weight was considered as a percentage of body weight, however, no significant effects of testosterone treatments were indicated.

3.4.1.4. Plasma Estradiol and Testosterone

The plasma estradiol and testosterone concentrations at the conclusion of the study for the four main study groups, the prepubertally ovariectomized females, are shown in Table IX. Within these ovariectomized female groups, the plasma estradiol levels did not differ according to treatment. Mean plasma testosterone was high in groups which had been treated with testosterone in adulthood, just prior to the

end of the study, indicating high circulating levels as expected. Plasma testosterone levels were significantly higher in the group which received both peripubertal and adult testosterone than in the group which received only adult testosterone. Plasma testosterone levels were not detectable in those groups which did not receive adult testosterone.

3.4.2. All Treatment Groups

3.4.2.1. Erythromycin Demethylase Activity

Erythromycin demethylase activity in all twelve treatment groups is presented in Table X and Figures 27 and 28, calculated on the basis of both microsomal protein and microsomal P450. Statistical analysis was done using two-way ANOVA (2 x 6, sex by treatment) followed by multiple comparison testing. Group variances were found to differ, however the ANOVA procedure is considered to be "robust" and relatively unaffected by nonhomogeneous variances when the number of cases per group is approximately equal, as in this study (Norusis, 1988). Consistent with this is the observation that when the data were transformed to reduce variance inequality, as shown in Appendix Tables D and E, the statistical results were not greatly different from the untransformed results. By all methods of calculation used,

both sex and treatment were significant and interactive factors, thus the erythromycin demethylase response to treatment depended upon the sex of the animal.

The results of multiple comparison testing are shown in Tables XI and XII. The following comparisons were of interest. Erythromycin demethylase activity was significantly higher in control males than in control females, 1.6 fold (by protein) and 1.5 fold (by P450). Nongonadectomized males and females responded differently to adult testosterone administration: erythromycin demethylase activity increased in the males but not in the females. This indicated the presence of an androgen response system in the adult males which was not observed in the adult female. Further, the increase in the males showed that the control males were not expressing erythromycin demethylase activity at their maximum potential level.

In the female rats, prepubertal ovariectomy appeared to decrease erythromycin demethylase activity by 37% (by protein) and 40% (by P450) from control female levels, however this difference was not statistically significant. This decrease became statistically significant when the group variances were made more homogeneous by data transformation (Appendix Tables D & E), The administration

of both peripubertal and adult testosterone to prepubertally-ovariectomized females resulted in an erythromycin demethylase activity which did not differ from that of control females, and reached 31% (by protein) and 29% (by P450) of control male levels.

In the male rats, prepubertal castration reduced erythromycin demethylase activity by 58% (by protein) and 51% (by P450), a statistically significant difference. The erythromycin demethylase activity in the prepubertally castrated males was the same as the erythromycin demethylase activity in the prepubertally ovariectomized females and in control females. Administration of testosterone to castrated males around the time of puberty resulted in an increase in erythromycin demethylase activity by 52% (by protein) and 31% (by P450) over castrated levels, however these levels were not significantly different. Similarly, adult testosterone administration alone in prepubertally castrated males increased mean erythromycin demethylase activity (45% by protein, 41% by P450) but the levels were not statistically different. The level of hepatic microsomal erythromycin demethylase activity in prepubertally castrated male rats treated with adult testosterone was significantly lower than that of control males and of control males treated with adult testosterone. The administration of both

peripubertal and adult testosterone to castrated males resulted in a significant increase in erythromycin demethylase activity over that of castrated males which did not receive testosterone, by 125% (by protein) and 83% (by P450). The mean erythromycin demethylase activity in those animals treated with both periods of testosterone did not differ from that of the control males, or with that of castrated males treated with adult testosterone. The combination treatment produced significantly higher mean erythromycin demethylase activity than seen in the group treated with pubertal testosterone only, when calculated on the basis of microsomal protein. The combination treatment also produced higher hepatic microsomal erythromycin demethylase activity in the prepubertally castrated males than in the prepubertally ovariectomized females.

The results for the prepubertally castrated male rats were analyzed statistically by two-way ANOVA (2x2, pubertal testosterone by adult testosterone) in order to compare these results with those of the ovariectomized females. By either calculation, the combined testosterone treatments did not have an interactive effect on hepatic microsomal erythromycin demethylase activity in the castrated male rats (p=0.499 (protein), p=0.732 (P450)), however each testosterone treatment alone had a significant effect

(p=0.004 and 0.007 (protein), p=0.023 and 0.004 (p450)).

3.4.2.2. *Microsomal P450, Liver P450 and Protein*

Since differences were observed in erythromycin demethylase results depending upon the calculation used, differences in P450 and protein concentrations were investigated. These results are presented in Table X. The microsomal P450 content was analysed by two-way ANOVA (2 x 6, sex by treatment). Both sex and treatment were significant factors. Microsomal P450 content was significantly higher following adult testosterone treatment of both control males and castrated males given pubertal testosterone in comparison with adult testosterone-treated gonadectomized females and males, castrated males or ovariectomized females treated with pubertal testosterone. These microsomal differences largely reflected changes in liver P450 content in response to both sex and treatment and the interaction of these two factors. In contrast, liver protein content was affected by sex only, with control male levels being significantly lower than those of control females and ovariectomized females given peripubertal testosterone or both testosterone treatments.

3.4.2.3. Body and Liver Weights

Mean animal body and liver weights at the conclusion of the study are shown in Tables X, XIII, and XIV. Both parameters displayed differences according to both sex and treatment. However, when liver weight was considered as a percentage of body weight, only treatment had a significant effect. The most consistent difference of those observed was a significant decrease in liver weight as a percentage of body weight following gonadectomy in both males and females compared with controls.

3.4.2.4. Plasma Estradiol

Individual estradiol levels were measured in plasma samples obtained from female and control male animals at the conclusion of the study, twenty-four hours after any injections. The mean plasma estradiol levels for these treatment groups are shown in Figure 29.

Plasma estradiol levels in normal Sprague Dawley female rats range from 20 to 330 pg/mL, progressively increasing to proestrous (Dohler & Wuttke, 1975). Control female levels in this study were at the lower end of this normal range. Plasma estradiol levels in ovariectomized groups were significantly lower than levels in the control female group,

thus confirming the expected effects of surgery. The lower estradiol levels in the ovariectomized females were comparable to the levels detected in the control males. The administration of testosterone in adulthood did not alter the plasma estradiol levels significantly.

3.4.2.5. *Plasma Testosterone*

Plasma testosterone levels from individual animals were measured in blood samples taken 24 hours after the last injection. The average results in each treatment group are shown in Figure 30.

Normal adult male rat levels of plasma testosterone range from less than 1 to approximately 7 ng/mL, and fluctuate widely; this is partly due to a daily trimodal rhythm (Bartke *et al.*, 1973; Mynson & Liu, 1973; Kalva & Kalra, 1977; Mocta *et al.*, 1978). Normal adult female Sprague Dawley rats have plasma testosterone levels on the order of 0.2-0.4 ng/mL (Dohler & Wuttke, 1975). Observed mean plasma testosterone levels in control males in this study were within the normal range, at the lower end, while female levels were below the detection limits of the assay.

Plasma testosterone levels were examined for verification of castration in the male treatment groups. This data was only

useful for confirmation of surgery in some of the prepubertally castrated male groups, since in all groups injected with testosterone days 81-90, just prior to blood sampling, the mean plasma testosterone levels were elevated, indicating the persistence of injected testosterone.

Three animals were removed from the study on the basis of plasma testosterone measurements outside the range of two standard errors of the mean for their group.

Other differences in plasma testosterone were of interest (Table XV). Administration of testosterone in adulthood to males or females in all cases resulted in significantly higher mean plasma testosterone levels than those of the control male group, although still within the normal range stated above. For plasma testosterone levels, the response to adult testosterone administration appeared to depend both on sex and on exposure to pubertal testosterone. Higher levels were detected in males and in animals which received pubertal testosterone injections. This data suggested a difference in handling of the injected testosterone related to these factors.

Further, the mean plasma testosterone levels in ovariectomized females were equivalent to those in

gonadectomized males.

3.4.2.6. Erythromycin Demethylase Activity versus Plasma Testosterone

The relationship between hepatic microsomal erythromycin demethylase activity and plasma testosterone levels was investigated, using data from all study animals. A significant correlation was found between erythromycin demethylase activity, when calculated on the basis of microsomal protein or P450, and plasma testosterone. Linear regression analysis fit the lines erythromycin demethylase (per protein)=0.41 + 0.07 (±0.013 SEM) x plasma testosterone (R^2=0.22, p=0.000, Figure 31) and erythromycin demethylase (per P450)=0.37 + 0.045 (±0.009 SEM) x plasma testosterone (R^2=0.21, p=0.000). The intercepts indicate that a portion of hepatic microsomal erythromycin demethylase activity was not related to plasma testosterone levels. A correlation was not detected between the ratio of plasma testosterone to plasma estradiol and hepatic microsomal erythromycin demethylase activity.

Table IX. Major study results in prepubertally ovariectomized female Sprague Dawley rats. Values are expressed as mean ± S.E.M. (n=9). These results are also presented in Table X. Abbreviations: EDM, erythromycin demethylase; per protein, per nmole formaldehyde/min/mg protein; per P450, per nmole formaldehyde/min/nmole P450; Fem, female; Gx, prepubertal ovariectomy; Ta, adult testosterone treatment; Tp, peripubertal testosterone treatment; ND, none detected. "*" indicates a significant difference from all treatment groups in that row (p<0.05). "**" indicates a significant difference from FemGxTa and Fem GxTp groups in that row (p<0.05).

	TREATMENT GROUPS			
	Fem Gx	Fem GxTa	Fem GxTp	Fem GxTpTa
EDM per protein	0.31±0.06	0.34±0.04	0.33±0.02	* 0.55±0.03
EDM per P450	0.26±0.02	0.32±0.03	0.33±0.03	* 0.47±0.04
mg Protein per g wet weight of liver	80±4	78±3	84±3	87±2
nmoles P450 per g wet weight of liver	95±5	82±4	87±6	** 103±3
nmoles P450 per mg protein	1.19±0.04	1.06±0.04	1.04±0.07	1.18±0.02
Body Weight(g)	346±5	360±10	328±5	350±12
Liver Weight(g)	10.6±0.3	11.5±0.3	10.5±0.2	11.6±0.5
Liv/Body %	3.07±0.06	3.19±0.05	3.19±0.07	3.31±0.19
Plasma Testosterone (ng/mL)	ND	* 2.6±0.3	ND	* 4.0±0.2
Plasma Estradiol (pg/mL)	19±1	18±1	16±1	15±1

Figure 24. Major Study: Erythromycin demethylase activity: calculated as nanomoles formaldehyde/min/mg microsomal protein in prepubertally ovariectomized female Sprague Dawley rats (mean ± standard error of the mean, n=9). "*" indicates a significant difference from all other treatment groups shown (p<0.05). Animals received the following treatments:

Fem Gx: gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

Fem GxTa: gonadectomy day 25, corn oil days 35-50, testosterone enanthate in corn oil 5.0 μmoles/kg/day days 81-90.

Fem GxTp: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, corn oil days 81-90.

Fem GxTpTa: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, testosterone enanthate in corn oil 5.0 μmoles/kg/day days 81-90.

Figure 25. Major Study: Erythromycin demethylase activity per P450 in prepubertally-ovariectomized female Sprague Dawley rats (mean ± standard error of the mean, n=9). "*" indicates a significant difference from all other treatment groups shown (p<0.05). Animals received the following treatments:

Fem Gx: gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

Fem GxTa: gonadectomy day 25, corn oil days 35-50, testosterone enanthate in corn oil 5.0 μmoles/kg/day days 81-90.

Fem GxTp: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, corn oil days 81-90.

Fem GxTpTa: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, testosterone enanthate in corn oil 5.0 μmoles/kg/day days 81-90.

ERYTHROMYCIN DEMETHYLASE
(nanomoles formaldehyde/min/nanomole P−450)

Figure 26. Comparison of erythromycin demethylase activity in the pilot study and major study in prepubertally ovariectomized female Sprague Dawley rats (mean ± standard error of the mean, 4 (pilot) or 9 (major) animals per group). Erythromycin demethylase activity is expressed either per protein (nanomoles formaldehyde/min/mg mmicrosomal protein) or per P450 (nanomoles formaldehyde/min/nanomole microsomal P450). "*" indicates a significant difference from Fem Gx, Fem GxTa, and Fem GxTp treatment groups ($p < 0.05$). Animals received the following treatments:

Fem Gx: gonadectomy day 25, corn oil days 35-50, corn oil days 81-90.

Fem GxTa: gonadectomy day 25, corn oil days 35-50, testosterone enanthate in corn oil 2.5 (pilot study) or 5.0 (major study) μmoles/kg/day days 81-90.

Fem GxTp: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, corn oil days 81-90.

Fem GxTpTa: gonadectomy day 25, testosterone enanthate in corn oil 5 μmoles/kg/day days 35-50, testosterone enanthate in corn oil 2.5 (pilot study) or 5.0 (major study) μmoles/kg/day days 81-90.

ERYTHROMYCIN DEMETHYLASE

FEMALE TREATMENT GROUP

Gx

Gx + Ta

Gx + Tp

Gx + TpTa

Pilot study (per protein)

Pilot study (per P-450)

Major study (per protein)

Major study (per P-450)
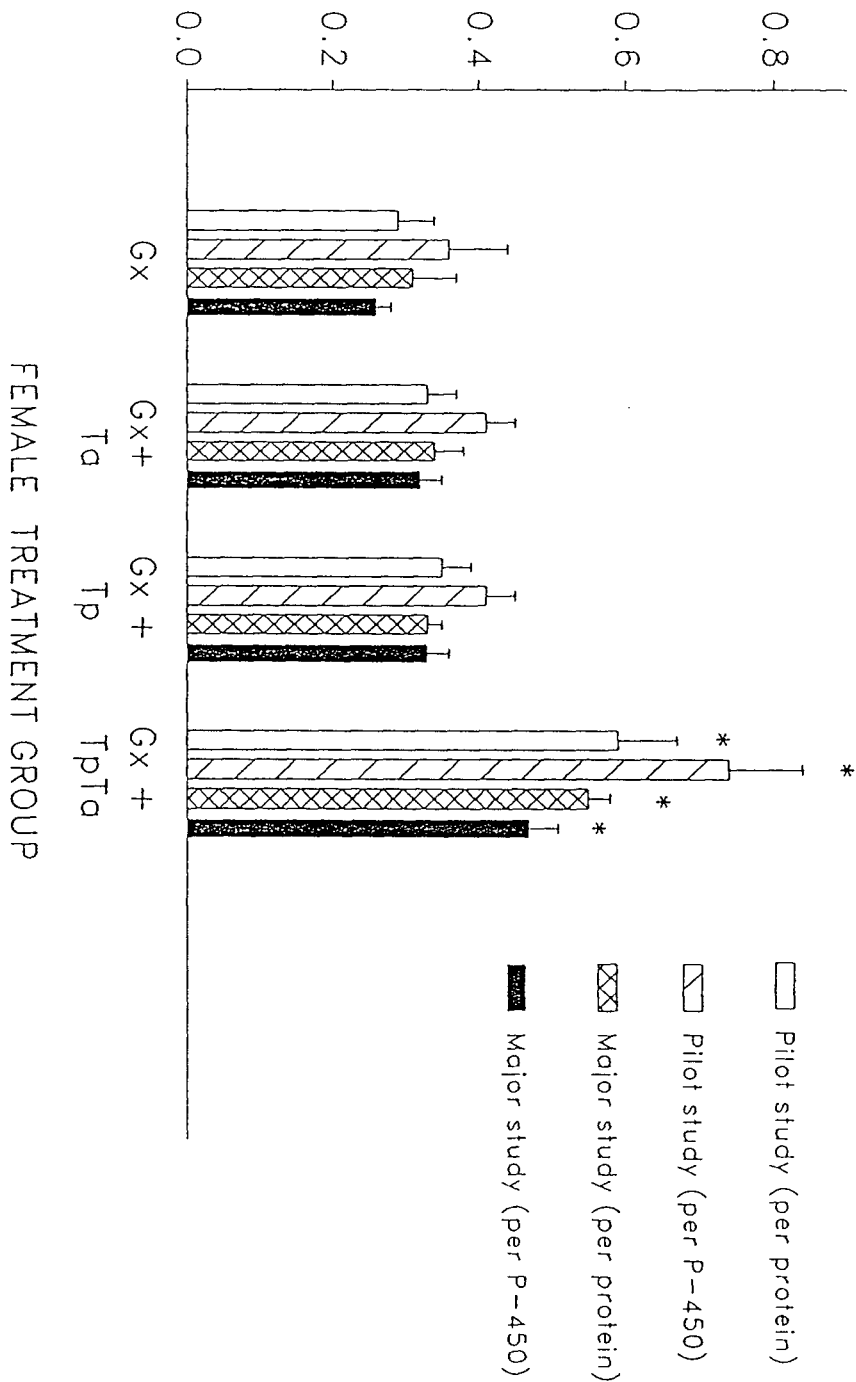
Table X. Results of major study, all treatment groups (mean ± S.E.M., 7-9 animals per group). Abbreviations: CON, control; Fem, female; Gx, prepubertal gonadectomy; Tp, peripubertal testosterone treatment; Ta, adult testosterone. †, for statistical analysis refer to Tables XI and XII. ††, for statistical analysis refer to Table XIII. †††, for statistical analysis refer to Table IV. "*" indicates a significant difference from CON MALE Ta group in that row (p<0.05); "&" indicates a significant difference from MALE GxTpTa group in that row (p<0.05); "**" indicates a significant difference from CON MALE group in that row (p<0.05); "+" indicates a significant difference from Fem Gx group in that row (p<0.05); "++" indicates a significant difference from MALE Gx group in that row (p<0.05).

Table X.

	CON Fem	CON Fem Ta	Fem Gx	Fem GxTa	Fem GxTp	Fem GxTpTa	CON MALE	CON MALE Ta	MALE Gx	MALE GxTa	MALE GxTp	MALE GxTpTa
TREATMENT GROUPS												
EDM $\dagger$ per protein	0.49 ±0.03	0.40 ±0.02	0.31 ±0.02	0.34 ±0.04	0.33 ±0.05	0.55 ±0.03	0.80 ±0.11	1.23 ±0.15	0.34 ±0.03	0.49 ±0.07	0.51 ±0.06	0.76 ±0.10
EDM $\dagger$ per P450	0.44 ±0.04	0.36 ±0.02	0.26 ±0.02	0.32 ±0.03	0.33 ±0.03	0.47 ±0.03	0.66 ±0.23	0.88 ±0.29	0.32 ±0.03	0.45 ±0.13	0.42 ±0.12	0.59 ±0.06
nmoles P450 per mg protein	* 1.13 ±0.05	* 1.12 ±0.03	* 1.19 ±0.04	*& 1.06 ±0.04	*& 1.04 ±0.07	* 1.18 ±0.02	>* 1.20 ±0.06	1.38 ±0.04	*& 1.06 ±0.02	*& 1.07 ±0.07	* 1.20 ±0.05	1.29 ±0.03
nmoles P450 per g wet weight of liver	97±4	93±4	95±5	* 82±4	* 87±6	103±3	* 87±7	110±5	* 82±3	* 85±5	93±6	100±3
mg protein per g wet weight of liver	** 86±2	82±3	80±4	78±3	** 84±4	** 87±2	72±2	79±8	78±3	80±1	77±3	77±1
Body Weight (g) $\dagger\dagger$	264±5	276±8	346±5	360±10	328±5	350±12	455±16	451±13	389±8	403±13	438±14	458±12
Liver Weight (g)$\dagger\dagger\dagger$	9.3±0.3	9.7±0.2	10.6±0.3	11.5±0.3	10.4±0.2	11.6±0.5	16.1±0.9	15.0±0.8	12.2±0.4	13.7±0.8	14.8±1.0	15.8±0.7
Liver/Body (%)	+ ++ 3.49 ±0.08	+ ++ 3.52 ±0.08	3.07 ±0.06	3.19 ±0.05	3.19 ±0.07	3.31 ±0.06	+ ++ 3.51 ±0.11	3.31 ±0.09	3.13 ±0.06	3.39 ±0.09	3.36 ±0.11	+ 3.45 ±0.08

Table XI. Major study: statistical analysis for erythromycin demethylase activity calculated per mg protein. "*" indicates pairs of groups which are significantly different (p<0.05). Treatment groups:
Grp 1: Con Fem
Grp 2: Con Fem Ta
Grp 3: Fem Gx
Grp 4: Fem Gx Ta
Grp 5: Fem Gx Tp
Grp 6: Fem Gx TpTa
Grp 7: Con Male
Grp 8: Con Male Ta
Grp 9: Male Gx
Grp 10: Male Gx Ta
Grp 11: Male Gx Tp
Grp 12: Male Gx TpTa
Abbreviations: Con, control; Gx, gonadectomy prepubertally; Fem, female; Tp, peripubertal testosterone; Ta, adult testosterone.

Mean	Group	G r p 3	G r p 5	G r p 9	G r p 4	G r p 2	G r p 1	G r p 1 0	G r p 1 1	G r p 6	G r p 1 2	G r p 7	G r p 8
.3094	Grp 3												
.3336	Grp 5												
.3382	Grp 9												
.3426	Grp 4												
.3993	Grp 2												
.4896	Grp 1												
.4913	Grp 10												
.5131	Grp 11												
.5472	Grp 6												
.7624	Grp 12	*	*	*	*	*			*	*			
.7996	Grp 7	*	*	*	*	*	*	*	*				
1.2324	Grp 8	*	*	*	*	*	*	*	*	*	*		

Table XII. Major study: statistical analysis for erythromycin demethylase activity calculated per nmole P450. "*" indicates pairs of groups which are significantly different (p<0.05). Treatment groups:

Grp 1: Con Fem
Grp 2: Con Fem Ta
Grp 3: Fem Gx
Grp 4: Fem Gx Ta
Grp 5: Fem Gx Tp
Grp 6: Fem Gx TpTa
Grp 7: Con Male
Grp 8: Con Male Ta
Grp 9: Male Gx
Grp 10: Male Gx Ta
Grp 11: Male Gx Tp
Grp 12: Male Gx TpTa

Abbreviations: Con, control; Gx, gonadectomy prepubertally; Fem, female; Tp, peripubertal testosterone; Ta, adult testosterone.

Mean	Group	Grp3	Grp9	Grp4	Grp5	Grp2	Grp11	Grp1	Grp10	Grp6	Grp12	Grp7	Grp8
.2632	Grp 3												
.3204	Grp 9												
.3223	Grp 4												
.3342	Grp 5												
.3569	Grp 2												
.4196	Grp11												
.4401	Grp 1												
.4528	Grp10												
.4656	Grp 6												
.5853	Grp12	*	*	*	*	*							
.6565	Grp 7	*	*	*	*	*	*	*	*	*			
.8825	Grp 8	*	*	*	*	*	*	*	*	*	*	*	

Table XIII. Major study: statistical analysis for body weights (g). "*" indicates pairs of groups which are significantly different (p<0.05). Treatment groups:
Grp 1: Con Fem
Grp 2: Con Fem Ta
Grp 3: Fem Gx
Grp 4: Fem Gx Ta
Grp 5: Fem Gx Tp
Grp 6: Fem Gx TpTa
Grp 7: Con Male
Grp 8: Con Male Ta
Grp 9: Male Gx
Grp 10: Male Gx Ta
Grp 11: Male Gx Tp
Grp 12: Male Gx TpTa
Abbreviations: Con, control; Gx, gonadectomy prepubertally; Fem, female; Tp, peripubertal testosterone; Ta, adult testosterone.

Mean	Group	Grp 1	Grp 2	Grp 5	Grp 3	Grp 6	Grp 4	Grp 9	Grp 10	Grp 11	Grp 8	Grp 7	Grp 12
263.7778	Grp 1												
275.5000	Grp 2												
328.0000	Grp 5	*	*										
345.7778	Grp 3	*	*										
349.7778	Grp 6	*	*										
360.0000	Grp 4	*	*										
388.5000	Grp 9	*	*	*	*	*							
403.1429	Grp 10	*	*	*	*	*	*						
438.2500	Grp 11	*	*	*	*	*	*	*	*				
450.8889	Grp 8	*	*	*	*	*	*	*	*				
454.8889	Grp 7	*	*	*	*	*	*	*	*				
458.0000	Grp 12	*	*	*	*	*	*	*	*				

Table XIV. Major study: statistical analysis for liver weights (g). "*" indicates pairs of groups which are significantly different (p<0.05). Treatment groups:
Grp 1: Con Fem
Grp 2: Con Fem Ta
Grp 3: Fem Gx
Grp 4: Fem Gx Ta
Grp 5: Fem Gx Tp
Grp 6: Fem Gx TpTa
Grp 7: Con Male
Grp 8: Con Male Ta
Grp 9: Male Gx
Grp 10: Male Gx Ta
Grp 11: Male Gx Tp
Grp 12: Male Gx TpTa
Abbreviations: Con, control; Gx, gonadectomy prepubertally; Fem, female; Tp, peripubertal testosterone; Ta, adult testosterone.

Mean	Group	Grp1	Grp2	Grp5	Grp3	Grp4	Grp6	Grp9	Grp10	Grp11	Grp8	Grp12	Grp7
9.2257	Grp 1												
9.6660	Grp 2												
10.4461	Grp 5												
10.6343	Grp 3												
11.4751	Grp 4												
11.6026	Grp 6	*											
12.1655	Grp 9	*											
13.7084	Grp10	*	*	*	*		*						
14.8131	Grp11	*	*	*	*	*	*	*					
14.9959	Grp 8	*	*	*	*	*	*	*					
15.8453	Grp12	*	*	*	*	*	*	*					
16.0616	Grp 7	*	*	*	*	*	*	*					

0

Figure 27. Major study: erythromycin demethylase activity calculated per protein for all treatment groups (mean ± standard error of the mean, 7-9 animals per group). Statistical analysis is shown in Table XI. Abbreviations: Con, control; Ta, adult testosterone days 81-90; Gx, gonadectomy prepubertally on day 25 of age; Tp, peripubertal testosterone days 35-50.

ERYTHROMYCIN DEMETHYLASE ACTIVITY
(nmoles formaldehyde/min/mg protein)
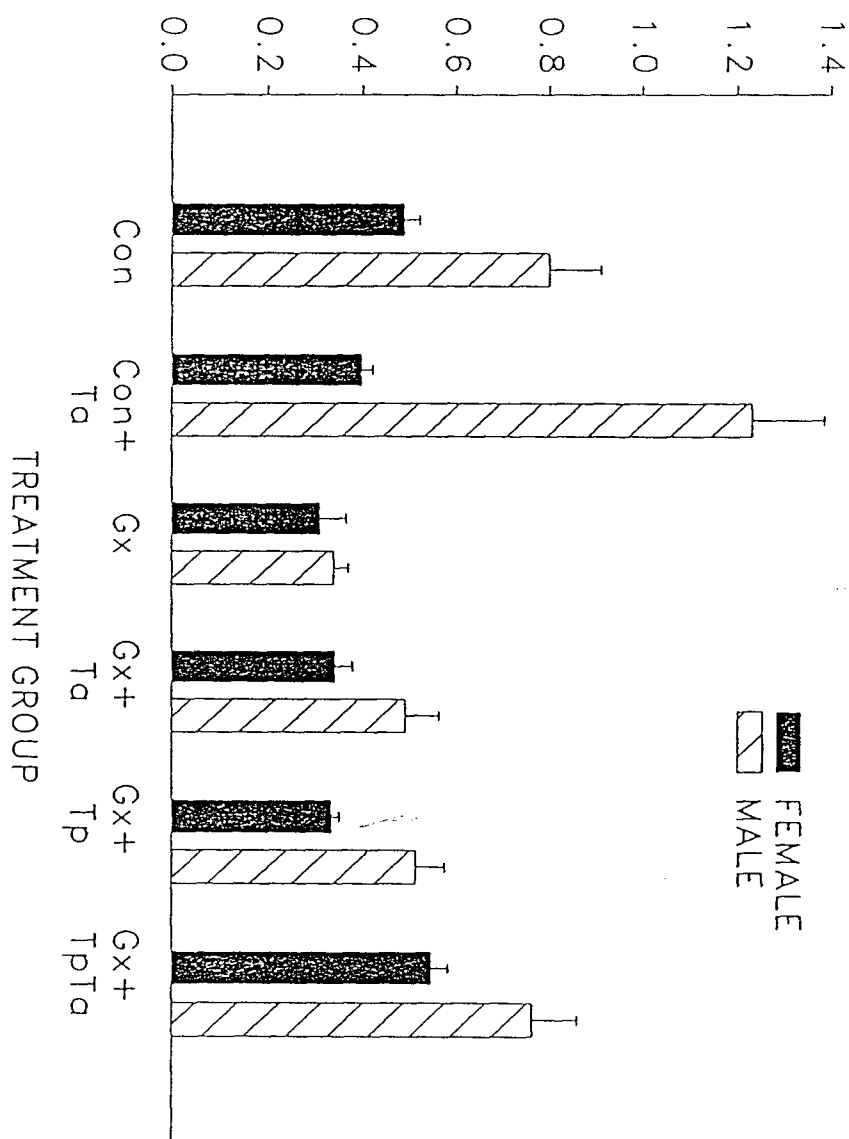
Figure 28. Major study: erythromycin demethylase activity calculated per P450 in all treatment groups (mean ±standard error of the mean, 7-9 animals per group). Statistical analysis is shown in Table XII. Abbreviations: Con, control; Ta, adult testosterone days 81-90; Gx, gonadectomy prepubertally on day 25 of age; Tp, peripubertal testosterone days 35-50.

ERYTHROMYCIN DEMETHYLASE ACTIVITY

(nmoles formaldehyde/min/nanomole P-450)
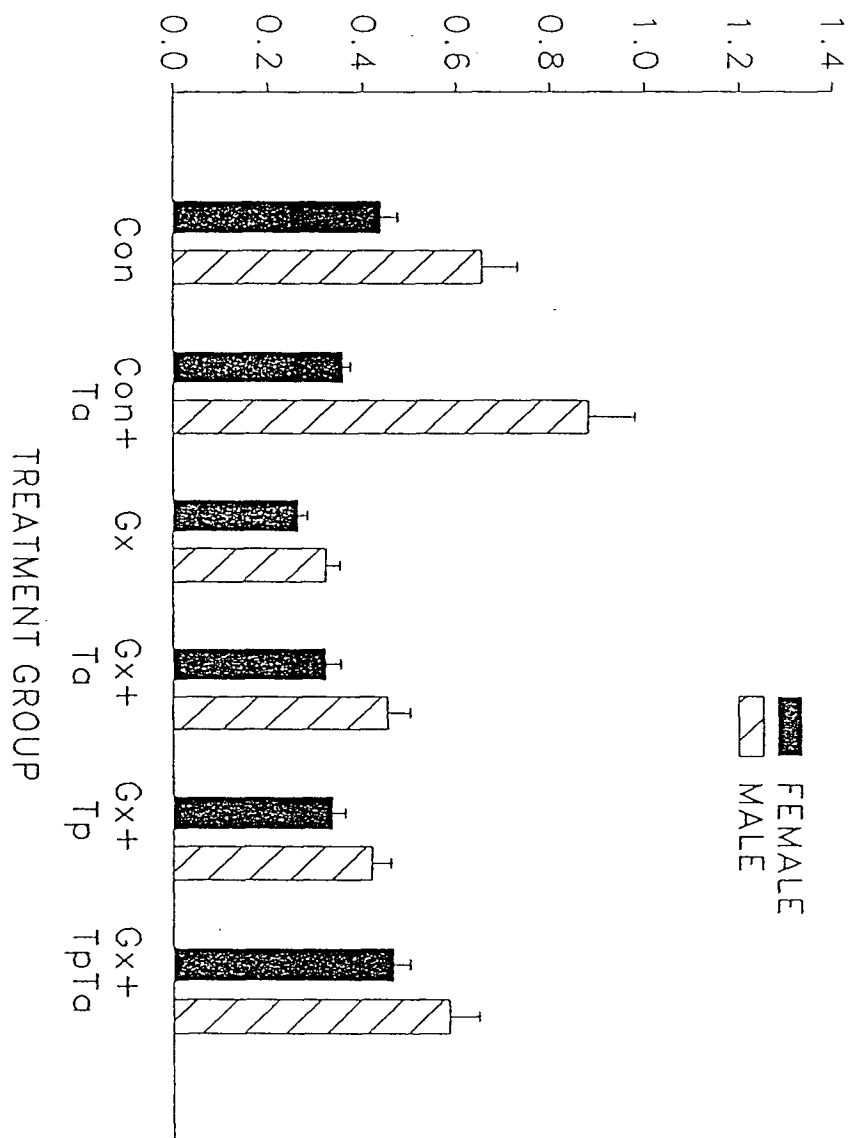
Figure 29. Major study: plasma estradiol levels (mean ± standard error of the mean, 7-9 animals per group). "*" indicates a significant difference from Con Fem (p<0.05). "**" indicates a significant difference from Con Fem Ta (p<0.05). Abbreviations: Fem, female; Con, control; Ta, adult testosterone days 81-90; Gx, gonadectomy prepubertally on day 25 of age; Tp, peripubertal testosterone days 35-50.
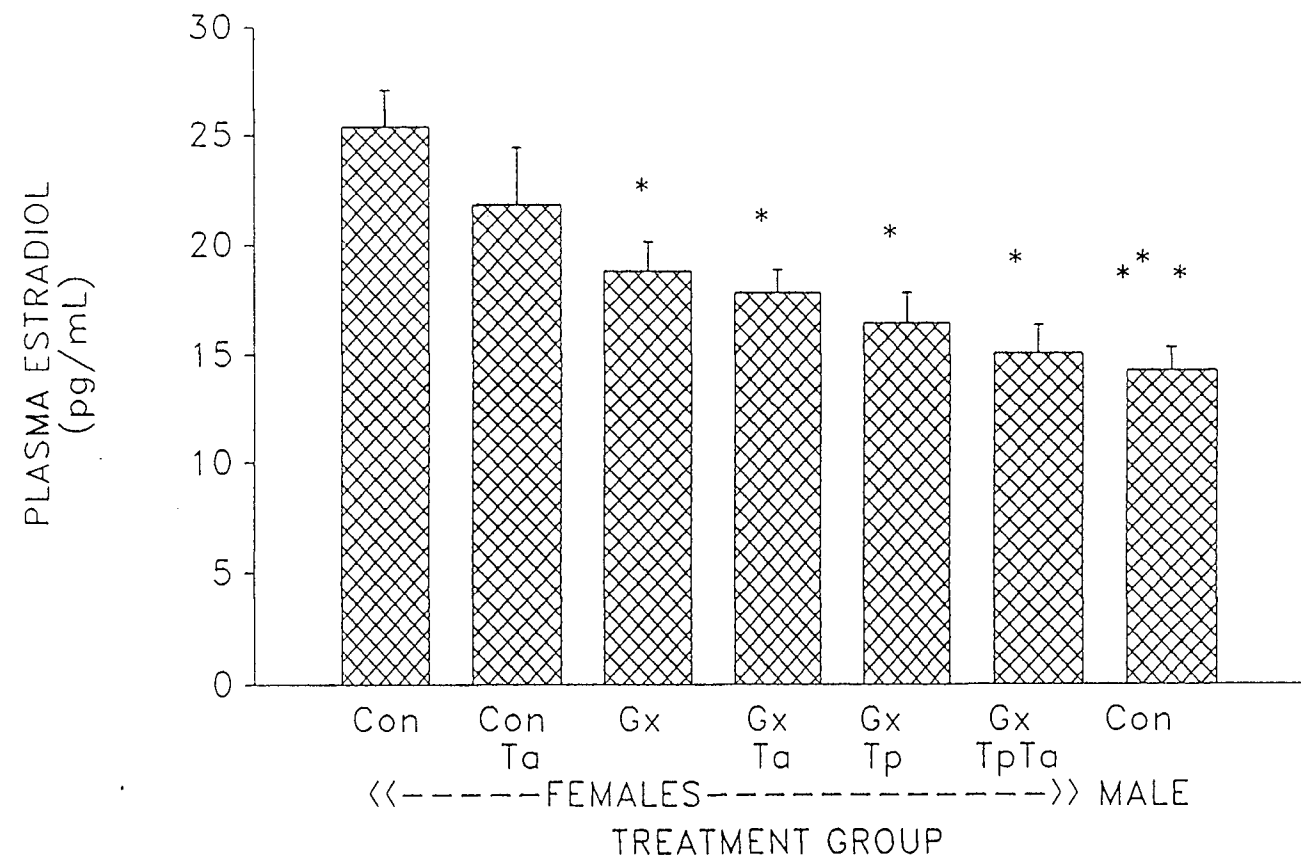
Figure 30. Major study: plasma testosterone concentrations (mean ± standard error of the mean, n=7-9 animals per group). Statistical analysis is presented in Table XIV. Abbreviations: Con, control; Ta, adult testosterone days 81-90; Gx, gonadectomy prepubertally on day 25 of age; Tp, peripubertal testosterone days 35-50, ND, not detectable.
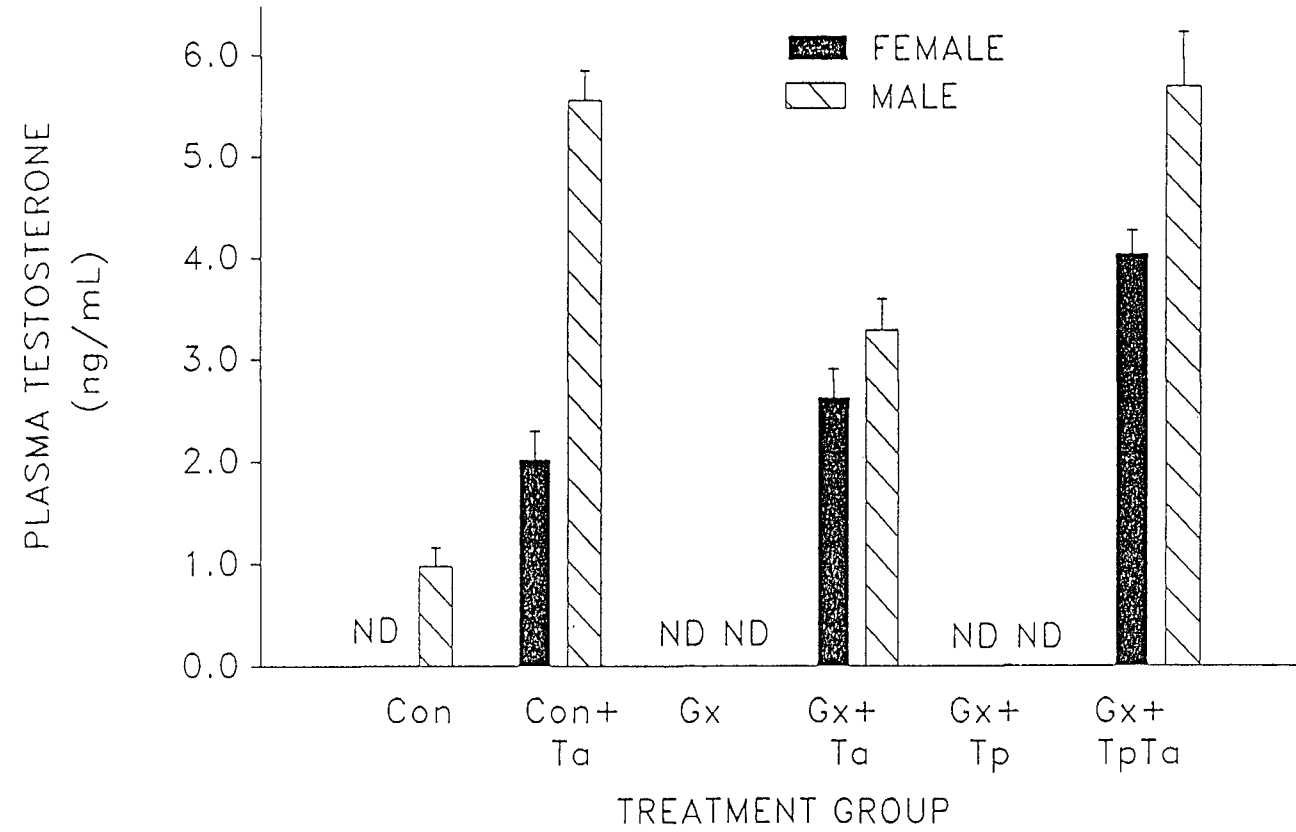
Table XV. Major study: statistical analysis for plasma testosterone concentrations. "*" indicates a significant difference between pairs of groups (p<0.05). Treatment groups:
Grp 1: Con Fem
Grp 2: Con Fem Ta
Grp 3: Fem Gx
Grp 4: Fem Gx Ta
Grp 5: Fem Gx Tp
Grp 6: Fem Gx TpTa
Grp 7: Con Male
Grp 8: Con Male Ta
Grp 9: Male Gx
Grp 10: Male Gx Ta
Grp 11: Male Gx Tp
Grp 12: Male Gx TpTa
Abbreviations: Con, control; Gx, gonadectomy prepubertally; Fem, female; Tp, peripubertal testosterone; Ta, adult testosterone.

Mean	Group	G r p 3	G r p 9	G r p 1	G r p 5	G r p 1 1	G r p 7	G r p 2	G r p 4	G r p 1 0	G r p 6	G r p 8	G r p 1 2
.0210	Grp 3												
.0265	Grp 9												
.0321	Grp 1												
.0712	Grp 5												
.0921	Grp 11												
.9800	Grp 7	*	*	*	*	*							
2.0233	Grp 2	*	*	*	*	*	*						
2.6257	Grp 4	*	*	*	*	*	*						
3.2945	Grp 10	*	*	*	*	*	*	*					
4.0398	Grp 6	*	*	*	*	*	*	*	*	*			
5.4454	Grp 8	*	*	*	*	*	*	*	*	*	*		
5.6978	Grp 12	*	*	*	*	*	*	*	*	*	*		

Figure 31. Erythromycin demethylase activity (nanomoles formaldehyde/min/mg protein) versus plasma testosterone (ng/mL).

PLASMA TESTOSTERONE
(ng/mL)

ERYTHROMYCIN DEMETHYLASE ACTIVITY
(nanomoles formaldehyde/min/mg protein)
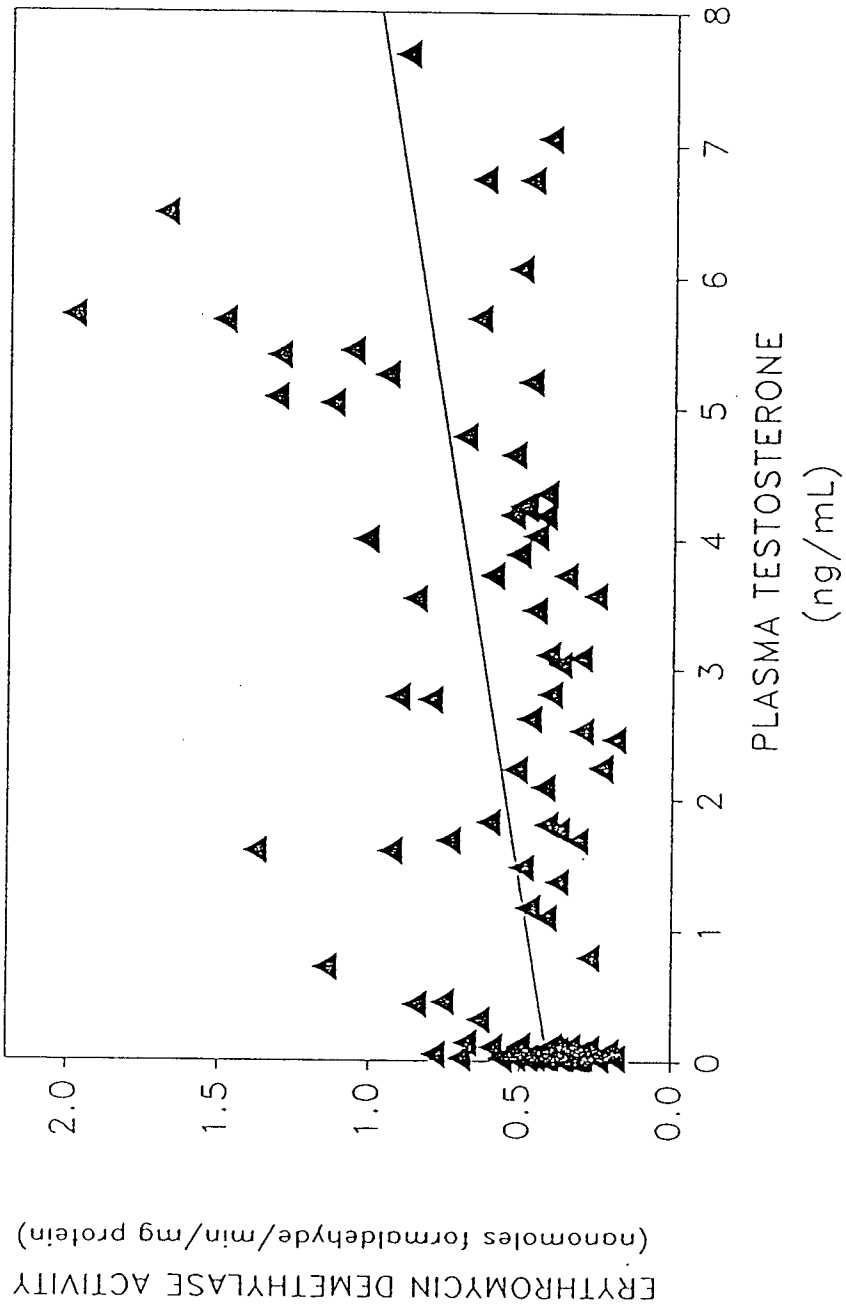
4. DISCUSSION

4.1. STUDY RESULTS

4.1.1. Non-gonadectomized Animals

Since this study had as its base the existence of a sex-dependent hepatic microsomal P450 enzyme activity, erythromycin demethylase, it was necessary to demonstrate a sex difference in non-gonadectomized animals for this parameter. We observed that hepatic microsomal erythromycin demethylase activity was higher, approximately 1.6-fold, in the adult male rat than in the adult female. The quality and magnitude of this sex difference were consistent with the previous report of hepatic microsomal erythromycin demethylase activity by Arlotto *et al.*. (1987). These two studies do not support that of Wrighton *et al.* (1985a), who reported slightly higher levels in females than in males.

The existence of responsiveness to androgen in the adult male rat for hepatic microsomal erythromycin demethylase activity is also required as a basis for this project. We observed a clear difference between intact males and females in the response to adult testosterone for this activity. An increased hepatic microsomal erythromycin demethylase activity was detected in non-gonadectomized male rats when

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they were treated with testosterone in adulthood, while the identical treatment did not increase erythromycin demethylase activity in the intact females. This indicated a second sex difference with respect to hepatic microsomal erythromycin demethylase activity, a lack of ability to respond to adult androgen in the intact female rat. This sex difference in adult androgen responsiveness has not been previously reported with respect to hepatic microsomal erythromycin demethylase activity. It is, however, consistent with our expectations, based on the observed sex difference in adult androgen responsiveness for another marker activity of the P450III family, steroid 6β-hydroxylase (Einarsson et al., 1973).

4.1.2. Effect of Prepubertal Gonadectomy

This study investigated hormonal effects in livers from prepubertally gonadectomized rats. Prepubertal ovariectomy of female rats decreased hepatic microsomal erythromycin demethylase activity by approximately 37 percent from intact female levels. Although this level of activity was not statistically different from that of intact females, the direction and magnitude of the change following ovariectomy were consistent in the pilot (38% decrease) and major (37% decrease) studies, arguing against a random effect. This observation was unexpected, and requires verification. If

true, it suggests that a portion of hepatic microsomal erythromycin demethylase activity in the adult female rat is dependent on the presence of ovarian factors, possibly estrogens.

In the males, we observed a statistically significant decrease of approximately 50 percent in hepatic microsomal erythromycin demethylase activity following prepubertal castration. The level of hepatic microsomal erythromycin demethylase activity in prepubertally castrated males was equivalent to that observed in prepubertally ovariectomized females and non-ovariectomized females, indicating that the sex difference required the presence of testes in the male after day 25 of age. Plasma testosterone levels were also decreased in the males by prepubertal castration, as expected. Although not specifically tested in this study, these observations, along with the observed sex difference in an adult androgen response potential discussed above, are also suggestive that high levels of circulating testosterone plus an ability to respond to that testosterone are required for the high hepatic microsomal erythromycin demethylase activity in the adult male rat.

4.1.3. Peripubertal Testosterone

The major purpose of the present study was to investigate the ability of peripubertal testosterone to influence the adult androgen responsiveness of an hepatic P450 enzyme activity in an ovariectomized female rat liver model. We observed that erythromycin demethylase activity in hepatic microsomes from prepubertally ovariectomized female rats increased in response to adult testosterone only if the animals had been exposed to peripubertal testosterone. This demonstrated that, in the ovariectomized female liver model used, peripubertal testosterone was capable of imprinting an adult androgen responsiveness for hepatic microsomal erythromycin demethylase activity. Statistical analysis, when based on activity per mg protein, supported the conclusion that an interactive effect was present; that is, the response of erythromycin demethylase activity in female rat liver to adult testosterone depended on whether or not pubertal testosterone exposure had occurred.

In the males, statistical analysis did not indicate an interactive effect between the two testosterone treatments. From the data, it is possible that each testosterone treatment had a relatively small but additive effect in the male livers, however statistically each treatment alone had no effect. This is an important point, since if the

potential for adult androgen responsiveness in the male is fully imprinted prior to puberty, for example neonatally, then males castrated at 25 days of age should show the same response to adult testosterone as the non-castrated rats. If such a result had been observed, then the biological relevance of pubertal imprinting by testosterone of adult androgen responsiveness for hepatic microsomal erythromycin demethylase activity would be questionable. We observed that the hepatic microsomal erythromycin demethylase activity level following adult testosterone in gonadectomized males was lower than that of intact males and of intact males given adult testosterone. Therefore, the magnitude of response to adult testosterone was smaller in males which had been castrated at 25 days of age than in intact males. These data indicated that neonatal testosterone had not permanently imprinted a complete adult androgen response potential for hepatic microsomal erythromycin demethylase activity in the male rat, and indicated a requirement for testicular factors after 25 days of age in the male for a full androgen response for this activity.

In the males, the effect of peripubertal testosterone following castration was similar but not identical with the effect observed in the females. Hepatic microsomal erythromycin demethylase activity was equivalent in

prepubertally-gonadectomized males and females, however subsequent combination testosterone treatment produced in the male case a level of hepatic microsomal erythromycin demethylase activity which did not differ from control male levels, while in the females the identical treatment resulted in an activity level equivalent to that of a control female, and lower than the control males. Therefore, the magnitude of the response in hepatic microsomal erythromycin demethylase activity to both peripubertal and adult testosterone was greater in the male than in the female livers following prepubertal gonadectomy. This greater magnitude of response suggests that peripubertal testosterone alone is not sufficient to produce in females the full androgen response seen in the males. This sex difference may indicate either the presence of inhibitory conditions, or a lack of required factors, in the ovariectomized female rat. These results are consistent with the possibility that prepubertal or neonatal testosterone might be the required factor absent in the female.

The results were not conclusive concerning the effects of single testosterone treatments following prepubertal castration in the male rats. Although a small response in hepatic microsomal erythromycin demethylase activity was observed to both adult or peripubertal testosterone alone in

the males, these responses did not differ statistically from those of the prepubertally ovariectomized females to these single treatments with testosterone.

4.1.4. Correlation with Plasma Testosterone

When plasma testosterone levels were determined in animals of both sexes and following the various treatments used in this study, we observed a positive correlation between hepatic microsomal erythromycin demethylase activity (by either calculation) and adult plasma testosterone level. The coefficients of determination were 0.22 and 0.21, therefore the degree of correlation was not large. This indicates that other factors are involved, or may relate to the fact that plasma testosterone is not a static parameter but rather shows wide individual diurnal variations (Bartke *et al.*, 1973; Kinson & Liu, 1973; Kalva & Kalra, 1977; Mock *et al.*, 1983). It is possible that hepatic microsomal erythromycin demethylase activity would correlate differently with free plasma testosterone levels, since circulating testosterone is usually highly bound to sex hormone-binding globulin (Gilman *et al.*, 1980). The observation that higher adult plasma testosterone levels correlated with higher hepatic microsomal erythromycin demethylase activity was in agreement with the observations made above which suggested that high hepatic microsomal erythromycin demethylase

activity in adult male rats is in part due to high circulating testosterone levels in the male. We observed an intercept for hepatic microsomal erythromycin demethylase activity of approximately 0.41 nmoles formaldehyde/min/mg protein (± 0.04 SEM), indicating a portion of the activity which was not dependent on plasma testosterone. As one would expect if this were so, the testosterone-independent level of hepatic microsomal erythromycin demethylase activity was similar to the average level observed in control adult females (0.49 ±0.03) and in castrated males (0.34 ±0.03). Therefore, there appeared to be a testosterone-independent basal level of hepatic microsomal erythromycin demethylase activity plus an additional testosterone-related component.

4.1.5. Comparison with BPH Studies

The only other demonstration of peripubertal imprinting of testosterone responsiveness for P450 enzymes has involved another sex-differentiated activity, hepatic microsomal BPH (Pak *et al.*, 1984; unpublished). It is therefore important to compare those observations with the results of the present study for hepatic microsomal erythromycin demethylase activity. Both activities are higher in adult male liver, however BPH in males is four times that in females (Wiebel & Gelboin, 1975; Pak *et al.*, 1984), while only a 1.6-fold difference has been observed for hepatic microsomal

erythromycin demethylase activity (Arlotto *et al.*, 1987). For both hepatic microsomal erythromycin demethylase activity and BPH, neither intact nor ovariectomized females responded to adult testosterone, indicating the lack of an adult androgen response system. For both activities, pubertal testosterone exposure has been shown to imprint an adult androgen responsiveness to testosterone in prepubertally ovariectomized female rats. However, two further differences between hepatic microsomal erythromycin demethylase activity and BPH were observed: (1) for BPH, combination testosterone treatment of prepubertally ovariectomized females resulted in BPH levels which were twice those of the control females, and represented 75 percent of control male levels. For hepatic microsomal erythromycin demethylase activity, similar treatment in females resulted in levels which were not greater than control female levels, and only 30 percent of control male levels. This is particularly striking since a larger adult dose of testosterone was used in the present study for hepatic microsomal erythromycin demethylase activity, yet a smaller response was observed. (2) Prepubertal ovariectomy did not alter BPH activity, while this treatment consistently reduced hepatic microsomal erythromycin demethylase activity. Therefore, it appears unlikely that the sex-dependent differences in BPH activity could be completely explained by the sex-related differences

represented by hepatic microsomal erythromycin demethylase activity. This was expected, since the studies of Kato *et al* (1987) and Ohgiya *et al* (1989) indicate that a large portion of BPH is related to P450h. Since hepatic microsomal erythromycin demethylase activity has been linked with P450p of the P450III family (Wrighton *et al.*, 1985a; Table IV), it is of interest to compare the results of this study with any similar P450p experiments. However, studies within the P450III family investigating androgen regulation have been conducted only for P450 Pb-2a/PCN-E (Waxman *et al.*, 1985; Dannan *et al.*, 1986), which is currently not considered to be the same as P450p (Hostetler, 1987; Table IV). In a study by Waxman *et al.* (1985), peripubertal castration (day 35) did not alter hepatic microsomal levels of P450 Pb-2a/PCN-E in adult male rats, in contrast to the 50 percent decrease in adult hepatic microsomal erythromycin demethylase activity observed in the present study following prepubertal castration (day 25). This suggests that either different parameters were being measured, or that the time period between days 25 and 35 of age was important. The study of Dannan *et al.* (1986) reported that neonatal castration completely removed detectable levels of P450 2a/PCN-E in adult male rats, however subsequent treatment with testosterone continuously throughout puberty and adulthood, restored these levels. The response in neonatally

ovariectomized females to continuous testosterone throughout puberty and adulthood was identical to that observed in the males. These results are somewhat similar to the results presented in this study, in that pubertal plus adult testosterone exposure produced an increase in the ovariectomized female for the parameter measured. However, the magnitude of the response in hepatic microsomal erythromycin demethylase activity in females was not as large as the response in males. Again, it is not clear if these differences resulted from differences in the study protocols, or from measuring different enzymes. It should also be noted that hepatic microsomal erythromycin demethylase activity was detectable in female rats in the present study, while P450p (Wrighton *et al.*, 1985a) and other P450III forms (Gozukara *et al.*, 1984; Waxman *et al.*, 1985; Cresteil *et al.*, 1986; Imoake *et al.*, 1988) are not detectable or present at very low levels in female rat hepatic microsomes. It is not possible to make definite conclusions concerning the regulation of the P450III family based on the results of the present study.

4.1.6. Relation to Gustafsson's Theory

The current concept of hormonal regulation of hepatic P450 enzymes is strongly based upon the theories of Gustafsson (Einarsson *et al.*, 1973; Gustafsson *et al.*, 1974a; Gustafsson *et*

al., 1974b; Gustafsson et al, 1983), which place an emphasis on neonatal imprinting by testicular androgens. Indeed, for a subgroup of hepatic P450s evidence has been presented in support of the hypothesis that neonatal androgens influence sex-dependent hepatic steroid metabolizing P450s via an indirect effect on pituitary growth hormone patterns (Jansson *et al.*, 1984; 1985a; 1985b; 1985c; Jansson & Frohman, 1987). However, this proposal allows that additional control mechanisms may exist, and that other P450s may be regulated in a different manner.

With respect to the time of imprinting of adult androgen responsiveness for sex-dependent P450s, evidence for the neonatal period is derived primarily from a single study done by Gustafsson *et al.* (1974b). In that study, an adult androgen responsiveness for the 6β-hydroxylation of 4-androstene-3,17-dione was removed in male rats castrated at birth, but present if castration was done at 14 days of age, indicating an imprinting in the first days of life. Since testosterone 6β-hydroxylase (Gonzalez *et al.*, 1986; Imaoka *et al.*,, 1988) and hepatic microsomal erythromycin demethylase activity (Wrighton *et al.*, 1985a) are both considered as markers for members of the P450III family, the report of Gustafsson et al (1974b) would appear to be in conflict with finding in the present study of pubertal

imprinting. However, it is possible that this discrepancy is due either to the complexity of the P450III family (Table IV), or to the use of a steroid substrate other than testosterone. Levin and co-workers (Swinney *et al.*, 1987) have shown that steroid hydroxylations by specific P450s can be substrate-specific. Other studies have investigated neonatal imprinting of a basal level and not imprinting of adult androgen responsivity. It is also possible that the potential for imprinting of adult androgen responsiveness may be present in both the neonatal and pubertal periods. It remains to be shown whether neonatal testosterone administration to female rats can result in partial or full adult androgen responsiveness for sex-differentiated P450s. Therefore, our results do not necessarily conflict with current theory, but rather constitute an extension of the theory to include other regulatory mechanisms and other P450 forms.

4.2. POTENTIAL STUDY LIMITATIONS

Several possible limitations should be considered when interpreting the results of this study.

4.2.1. Erythromycin Demethylase Assay

4.2.1.1. Specificity

Research in the area of the P450 enzymes is currently at the stage where the properties of specific enzyme forms are being investigated (Tables I to III). In the present study, the erythromycin demethylase assay has been used as a marker activity for the P450III family. Is it possible to link erythromycin demethylase activity with a single specific P450 form? In fact, it is not clear which P450 form or forms are represented by hepatic microsomal erythromycin demethylase activity. A link with the P450III family has been made by the observation that hepatic microsomal erythromycin demethylase activity is inhibited by antibodies against P450p (Wrighton *et al.*, 1985a). In that study, polyclonal antibodies, prepared against P450p which had been purified from livers from PCN or dexamethasone treated female rats, and made form-specific by back-absorption against microsomes prepared from rats treated with 3-methylcholanthrene (Elshourbagy *et al.*, 1981), were shown to block approximately 75 percent of the erythromycin demethylase activity in liver microsomes from adult female Sprague Dawley rats which had been treated with TAO, PCN or dexamethasone. Therefore, while P450p appears to play a major role in hepatic microsomal erythromycin demethylase

activity in the induced female rat, the identity of the enzyme(s) responsible for the remaining erythromycin demethylase activity which was not blocked by anti-P450p antibodies remains unknown. It has, as well, not been proven that P450p is a single enzyme. Further, this immunoinhibition study concerns only the induced situation in female rats; it is not known if antibodies which react with P450III forms found in untreated male and female rats will inhibit hepatic microsomal erythromycin demethylase activity in untreated animals. Although it has not been shown, it is quite possible that different P450 forms demethylate erythromycin in male and female rats, and in untreated and induced rats. The works of Gonzalez *et al.* (1986), for example, identified PCN/Dex-induced P450III forms which were undetectable in untreated rats.

Enzyme kinetic studies have indicated the presence of at least two enzymes catalyzing hepatic microsomal erythromycin demethylase activity in untreated female Long Evans rats (Arlotto *et al.*, 1987). In the present study, a detailed investigation of the enzyme kinetics of the erythromycin demethylase activity in Sprague Dawley rats was not attempted. The limited data were inconclusive, allowing for the possibility of either single or multiple enzymes catalyzing this reaction.

Other evidence correlating erythromycin demethylase activity with P450III activity in hepatic microsomes has included the observations that (i) PCN induces both erythromycin demethylase activity (Wrighton *et al.*, 1985a), P450Pb-2a/PCN-E (Waxman *et al.*, 1985) and P450p (Hostetler *et al.*, 1987); and (ii) both hepatic microsomal erythromycin demethylase activity (Arlotto *et al.*, 1987) and immunochemically-detectable levels of P450 Pb-2a/PCN-E (Gozukara *et al.*, 1984; Waxman *et al.*, 1985) and P450 Pb-1 (Imaoka *et al.*, 1988) have been reported as higher in hepatic microsomes from adult male rats than from adult female rats; and (iii) in the female rat, hepatic microsomal erythromycin demethylase activity appears to decrease with age (Arlotto *et al.*, 1987) as do immunochemically-quantitated levels of P450 Pb-2a/PCN-E (Waxman *et al.*, 1985) and P450 Pb-1 (Imaoka *et al.*, 1988; Yamazoe *et al.*, 1988). However, in the study by Wrighton *et al.* (1985a), P450p levels did not correlate with hepatic microsomal erythromycin demethylase activity in untreated rats, since while P450p was detectable in hepatic microsomes from the untreated males but not from the females, the hepatic microsomal erythromycin demethylase activity was not only detectable in females, but higher in the females than in the males. P450III forms, unlike erythromycin demethylase, are present at only low or undetectable levels in hepatic microsomes from adult female

rats (Gozukara *et al.*, 1984; Waxman *et al.*, 1985; Cresteil *et al.*, 1986; Imaoka *et al.*, 1988).

Therefore, although hepatic microsomal erythromycin demethylase activity has been linked with the P450III forms, due to the reports of multiple forms of P450 catalyzing the erythromycin demethylase reaction, and the fact that all of these forms have not yet been identified, it is not possible to conclude that changes in hepatic microsomal erythromycin demethylase activity reported in this study were directly correlated with changes in a P450III form. The possible existence of multiple forms as components of erythromycin demethylase activity further complicated the interpretation of any results, since, for example, if an increase in net activity is recorded, it is not clear whether one or more than one contributing enzyme has been affected. Similarly, a lack of net change in such a non-specific, multi-component enzyme activity may indicate, rather than a lack of effect, a positive effect on one component balanced against a negative effect on a second contributing component.

4.2.1.2. Sensitivity and Detection Limits
The sensitivity of an analytical method can be defined as the ratio of the change in response (analytical signal) to the change in the quantity or concentration of analyte being

measured (Massart, 1978; Ewing, 1985). In some branches of science, "sensitivity" has also been used to describe the detection limit of a method (Massart, 1978).

The erythromycin demethylase assay used in this study is based on the spectrophotometric measurement of formaldehyde according to the method of Nash (1953). Spectrophotometric methods are relatively insensitive analytical procedures, in comparison with fluorimetry (Ahuja, 1986), radiometric assays or HPLC (Smith & Stewart, 1981). As well, the method of Nash (1953) uses the Hantzsch reaction between acetylacetone, ammonia and formaldehyde. This reaction is not specific, and may detect non-formaldehyde substrates as well as endogenous formaldehyde in the absence of exogenously-added substrate or enzyme (Kleeberg & Klinger 1982). The result is a high "noise" (blank) level, which adversely affects the limits of detection. In this study, at the lowest observed erythromycin demethylase activity (the ovariectomized female rat group), the signal-to-noise ratio (absorbance reading/blank reading) was approximately 2:1. We were therefore measuring very close to the limits of detection of the assay (Massart, 1978). However, at the observed signal-to-noise ratio of 2:1, and with the standard deviation of the blank readings which represented 20% of the average blank readings in the female, these lowest

measurements were outside of three standard deviations of the blank mean and therefore distinguishable statistically from blank readings (Massart, 1978).

In terms of sensitivity as a measure of response to a change in the concentration being measured, the low sensitivity of spectrophotometric methods does not change the results in proportion to each other for hepatic microsomal erythromycin demethylase activity in the different treatment groups. However, it may have decreased the ability of the method to detect small differences between treatment groups.

4.2.1.3. Definition of Puberty

Puberty has been defined as the time at which gametogenesis and gonadal hormone secretion occurs, secondary sexual characters and reproductive functions begin, and sexual dimorphism is accentuated (Stedman, 1976). In the present study, days 35-50 of age were chosen for peripubertal testosterone treatment, in order to coincide with previous studies by Pak *et al.* (1984; unpublished) on peripubertal effects. This time period is consistent with statements in the literature that puberty occurs in male and female rats at between 40 to 60 days of age (Farris *et al.*, 1950; Baker *et al.*, 1980; Fax *et al.*, 1984; Harkness & Wagner, 1983). Forest (1979) has clearly shown a rise in plasma testosterone

levels in male Sprague Dawley rats at approximately 35 days of age. Therefore, although it cannot be ruled out that a pubertal testosterone exposure might have occurred unusually early in some of the study rats, perhaps prior to castration at 25 days of age, such an occurrence appears improbable.

4.2.1.4. Testosterone Dose

It is important to consider whether the doses of testosterone used in this study were physiological. The dose of testosterone used in the major study was that which had been shown to maintain "normal levels of sex accessory tissue weights and sex-dependent drug-metabolizing enzyme activities in gonadectomized animals" (Pak *et al.*, 1984). Plasma testosterone levels were within the physiological range as quoted in the literature (Dohler & Wuttke, 1975), and could therefore be considered approximately physiological. However, it is important to note that the pattern of plasma testosterone produced by daily exogenous injections would probably have differed from that of the normal male rat. Large fluctuations in plasma testosterone levels in male rats have been reported (Bartke *et al.*, 1973; Kalva & Kalra, 1977). In adult male rats, a circadian rhythm is seen; in male Sprague Dawley rats at 40-50 days of age, a trimodal rhythm has been reported with peak levels at 0200, 1200 and 1800 hours on a 12 hour light, 12 hour dark cycle

(Mock *et al.*, 1978). The injections used in the present study were of testosterone enanthate ester in corn oil, given once daily subcutaneously. Testosterone enanthate is more fat-soluble than testosterone, is more slowly absorbed and has a longer half-life than testosterone (Gilman *et al.*, 1980). In humans it is injected every one to two weeks for replacement therapy (Gilman *et al.*, 1980). It would therefore tend to produce a sustained, unfluctuating level of testosterone in the circulation, and certainly a plasma level which would not be regulated by normal physiological factors. The effects of testosterone administration observed in this study may therefore not correspond to normal physiological events in the developing rat.

The dose of testosterone administered to adult animals was twice as large in the major study as in the pilot study. In comparable treatment groups, higher erythromycin demethylase activity was not observed with the higher testosterone dose (Figure 26). This indicated that the effect was already maximal at the lower dose.

4.2.1.5. Calculations of Erythromycin Demethylase Activity

Hepatic microsomal erythromycin demethylase activity was calculated both on the basis of microsomal protein and on microsomal total P450. The patterns of erythromycin

demethylase activity in the study groups were similar for the two methods of calculation, however small differences did occur which altered the statistical results. For example, within the ovariectomized female groups, a significant interaction between the two testosterone treatments was detected using calculations based on protein, but not from the calculations based on total P450. Analysis of liver protein and P450 content for these four study groups revealed that the treatments had affected the liver P450 content but not the liver protein content (Table IX). Therefore, within these four groups, it can be argued that protein provided[0] a more stable and therefore more appropriate baseline for comparisons between groups. As well, P450 activities including erythromycin demethylase are generally presented in terms of product formation rate per mg microsomal protein (Wrighton *et al.*, 1985a; Arlotto *et al.*, 1987). Since control liver microsomes generally contain approximately 1 nmole total P450 per mg protein, and P450s have molecular weights of approximately 50,000 Da, it is clear that only approximately 1/20 (by weight) of the protein in liver microsomes is composed of P450. It can therefore be reasoned that microsomal protein would be relatively insensitive to any effect of the study treatments on the total P450 content of the liver.

Hepatic microsomal erythromycin demethylase activity calculated on the basis of total P450 has a different meaning, referring to the rate of product formation per nmole microsomal total P450. This calculation will be very sensitive to changes in total P450 content in the liver, and could give rise to anomolous results. For example, if the study treatments inhibit the synthesis of a P450 enzyme which is not being measured in the enzyme assay, but do not affect the P450 form which is being measured in the assay, the resultant decrease in total P450 will result in an apparent increase in the activity being studied. In this study, microsomal P450 content per mg protein decreased in the groups Fem GxTa and Fem GxTp (Table VIII) due to a relative decrease (8-13%) in total liver P450 in these groups. Although small and not statistically significant, this decrease may explain the apparent increase in hepatic microsomal erythromycin demethylase activity in these groups when calculated on the basis of P450 as compared to calculation on the basis of protein.

Therefore, these two calculations have different meanings, and can best be interpreted by consideration of the changes in liver P450 and protein. In this study, these effects were generally small, and did not alter the pattern of results. The largest change observed was a 15 percent increase in

total microsomal P450 per mg protein in non-gonadectomized males treated with adult testosterone. In addition, these effects were not consistent between the pilot and major studies, which tends to indicate random events.

There remains the question of whether changes in hepatic microsomal erythromycin demethylase activity indicate changes in the amount of an enzyme (or enzymes) catalyzing the reaction, or a change in the enzyme forms present, with altered product formation being due to changes in Km and Vmax. The present study did not attempt to distinguish between such quantitative and qualitative effects, therefore no firm conclusions can be made. It is possible that both qualitative and quantitative effects are involved.

4.3. SPECULATION ON RELEVANCE TO HUMANS

This study would be incomplete without a brief mention of its potential relevance to the human species. Is there a sex difference in hepatic microsomal P450s in humans, and if so, is it regulated by hormones?

As in rats, multiple forms of P450 have been identified in human liver (Guengerich 1987; Gonzalez, 1989), however quantitative or qualitative sex differences in specific human P450 forms have not been reported. It is not known if

this indicates a lack of sexually-differentiated P450 forms, or reflects the current paucity of human data.

The possibility of a sex difference in human steroid metabolism has been investigated. Thrasher *et al.* (1969) found a higher urinary 6β-hydroxycortisol/17-hydroxycorticosteroid ratio in females. Pfaffenberger and Horning (1977) have also reported a sex difference in human steroid metabolism, based on an analysis of urinary steroid metabolites. However, human liver studies of testosterone hydroxylation have not reported a sex difference, with 6β-hydroxytestosterone being the major hydroxylated metabolite formed (Lisboa and Gustafsson, 1968; Kremers *et al.*, 1981). As reviewed by Giudicelli & Tillement (1977) and Proksch & Lamy (1977), sex differences in drug metabolism have been reported for only a few compounds. The clearances of diazepam and prednisolone are higher in adult females (Ochs *et al.*, 1981; Meffin *et al.*, 1984), while the clearances of chlordiazepoxide and propranolol are higher in adult males (Roberts *et al.*, 1979; Walle *et al.*, 1989). These reported differences require verification, and have not been linked with differences in specific P450 forms. Information is even more sparse on regulation by hormones of sex differences, although hormonal influences on the metabolism of some drugs have been reported (Proksch & Lamy, 1977).

Metabolic evidence therefore suggests that sexually-differentiated P450 forms might eventually be identified in humans. If so, several speculations of pharmacological interest can be made. It is possible that the appropriate dosing of drugs with low therapeutic indices might vary between the sexes. The assessment of potential risk from toxic chemicals may also require a consideration of gender. And finally, if it is found that hormones regulate a sex difference in human P450 forms as in rats, then it is possible that hormonal influences could alter hepatic metabolism. Diseases which alter hormone levels, such as hypogonadism, Stein-Levinenthal syndrome and growth hormone secretion abnormalities, could have an influence on metabolism. Drugs which alter hormone levels, for example antiepileptic drugs and glucocorticoids which reduce free testosterone levels (Dana-Haeri *et al.*, 1982; MacAdams *et al.*, 1986) could also alter metabolism. These effects of drugs and disease on metabolism might be immediate, for example in adulthood. As well, if present during an early and sensitive developmental period, drug or disease-related hormonal changes could permanently alter a later metabolic potential, long after the disease had been ameliorated or the drug cleared from the body.

4.4. PROPOSALS FOR FUTURE RESEARCH

In order to extend the results of the present study, several directions for future research are proposed.

The interpretation of the results of this study was limited by the lack of specificity of the erythromycin demethylase assay. It is therefore essential to conduct future research using methods which can be linked as much as possible to specific P450 forms. The measurement of regio- and stereo-selective testosterone hydroxylation by HPLC, or, preferably, if available, the use of mono-specific antibodies is required. The results of this study indicated that P450III forms should be further investigated with respect to pubertal imprinting of androgen responsiveness. As well, for the reasons outlined in the introduction, the possibility that pubertal testosterone could imprint androgen responsiveness for P450h should be investigated. Such a possibility is particularly exciting, since much of the research emphasizing the importance of the *neonatal* period for androgen imprinting has involved P450h. Therefore, a study using antibodies specific for P450III forms and P450h to investigate the imprinting of adult androgen responsiveness is suggested.

Secondly, the timing of the "critical period" during which

adult androgen responsiveness can be imprinted by testosterone requires further clarification. Neonatal gonadectomy and subsequent testosterone administration at discrete intervals ranging from birth through puberty and into adulthood would provide further evidence regarding the relative importance of the neonatal, pubertal, and later periods for the imprinting of this parameter.

Ultimately, the mechanisms involved in the imprinting of adult androgen responsiveness require elucidation. The combination of enzyme kinetic studies with immunoquantitation of specific P450 levels would distinguish qualitative versus quantitative effects. Studies in both ovariectomized and non-ovariectomized female rats would indicate any possible role of the ovaries in the inhibition of pubertal testosterone imprinting. A possible indirect effect of testosterone on the liver via growth hormone should also be investigated. For example, an experiment similar to the present one, investigating the effects of intermittent, male pattern, growth hormone administration during puberty on adult androgen responsiveness could be conducted.

4.5. SUMMARY AND CONCLUSIONS

We conclude the following:

1. Hepatic microsomal erythromycin demethylase activity was higher in adult male rats than in adult females, further corroborating the findings of Arlotto *et al.* (1987) that this is an hepatic P450 enzyme activity which is sexually-differentiated.

2. Adult male rats (non-castrated) but not adult female rats (non-ovariectomized) responded to adult testosterone treatment at physiological doses with an increase in hepatic microsomal erythromycin demethylase activity. Therefore, the intact adult female rat lacked the androgen responsiveness with respect to hepatic microsomal erythromycin demethylase activity which was present in the adult male rat.

3. Prepubertal castration of male rats reduced hepatic microsomal erythromycin demethylase activity and plasma testosterone levels from control levels. Hepatic microsomal erythromycin demethylase activity in the different treatment groups was found to be partially correlated with plasma testosterone levels. These data suggested that the higher hepatic microsomal erythromycin demethylase activity in the adult male rat is related to the high adult male levels of circulating testosterone.

4. In adult female rats which had been ovariectomized prepubertally, exposure to testosterone peripubertally resulted in an adult responsiveness to testosterone for hepatic microsomal erythromycin demethylase activity. This indicated that the potential is present in the prepubertally-ovariectomized female rat for the pubertal imprinting by testosterone of an adult androgen responsiveness for hepatic microsomal erythromycin demethylase activity.

5. The administration of testosterone to adult male rats which had been prepubertally castrated resulted in hepatic microsomal erythromycin demethylase activity which was lower than that of intact males and of intact males treated with testosterone in adulthood. This result indicated that adult androgen responsiveness of hepatic microsomal erythromycin demethylase activity is not completely imprinted in males in the neonatal period.

6. The magnitude of the adult androgen response for hepatic microsomal erythromycin demethylase activity following peripubertal testosterone treatment of prepubertally-gonadectomized rats was larger in the male than in the female. These results suggested that peripubertal testosterone is not the sole factor influencing

the imprinting of adult androgen responsiveness for hepatic microsomal erythromycin demethylase activity.

7. The results were inconclusive concerning the role of pubertal testosterone in the imprinting of an adult androgen responsiveness for hepatic microsomal erythromycin demethylase activity in the prepubertally-castrated male rat. This indicated that factors other than pubertal testosterone were also involved.

8. This study provided evidence in support of the hypothesis that the pubertal period is a potential testosterone imprinting period for adult androgen responsiveness of hepatic P450 enzymes, and indicated that further research should be done in this area.

9. The results for hepatic microsomal erythromycin demethylase activity in this study could not definitely be linked to the P450III family of enzymes. However, since hepatic microsomal erythromycin demethylase activity has not been linked with any other P450 enzymes other than those of the P450III family, these results indicated that further studies of pubertal imprinting by testosterone of adult androgen responsiveness for P450III forms should be conducted using more specific techniques.

5. REFERENCES

Ahuja S, *Ultratrace Analysis of Pharmaceuticals and Other Compounds of Interest.* John Wiley & Sons, New York, 1986, p. 236.

Al-Dabagh SG, Idle JR and Smith RL, Animal modelling of human polymorphic drug oxidation - the metabolism of debrisoquine and phenacetin in rat inbred strains. *Biochem Pharmacol* **33:** 161-164, 1980.

Al-Turk WA, Stohs SJ and Roche EB, Effect of hypophysectomy and castration on hepatic, pulmonary and intestinal aryl hydrocarbon hydroxylase activities in the rat. *J Pharmacol Exp Ther* **216:** 492-495, 1981.

Arlotto MP, Sonderfan AJ, Klaassen CD and Parkinson A, Studies on pregnenolone-16a-carbonitrile-inducible form of rat liver microsomal cytochrome P450 and UDP-glucuronosyltransferase. *Biochem Pharmacol* **36:** 3859-3866, 1987.

Arlotto MP, Greenway DJ and Parkinson A, Purification of two isozymes of rat liver microsomal cytochrome P450 with testosterone 7a-hydroxylase activity. *Arch Biochem Biophys* **270:** 441-457, 1989.

Arlotto MP, Parkinson A, Identification of cytochrome P450a (P450IIA1) as the principal testosterone 7a-hydroxylase in rat liver microsomes and its regulation by thyroid hormones. *Arch Biochem Biophys* **270:** 458-471, 1989.

Aussel C, Uriel J and Mercier-Bodard C, Rat alpha-fetoprotein: isolation, characterization and estrogen-binding properties. *Biochimie* **55:** 1431-1437, 1973.

Baker HJ, Lindsay JR and Weisbroth SH, *The Laboratory Rat, Volume II.* Academic Press, New York, 1980, p 257.

Bandiera S, Ryan DE, Levinin W and Thomas PE, Evidence for a family of four immunochemically related isozymes of cytochrome P450 purified from untreated rats. *Arch Biochem Biophys* **240:** 478-482, 1985.

Bandiera S, Ryan DE, Levinin W and Thomas PE, Age- and sex-related expression of cytochromes P450f and P450g in rat liver. *Arch Biochem Biophys* **248:** 658-676, 1986.

Bartke A, Steele RE, Musto N, and Caldwell BV, Fluctuations in plasma testosterone levels in adult male rats and mice. *Endocrinology* **92:** 1223-1228, 1973.

Beach FA, Hormonal modification of sexually dimorphic behavior. *Psychoneuroendocrinology* **1:** 3-23, 1975.

Berg A and Gustafsson J-A, Regulation of hydroxylation of 5α-androstane-3α,17β-diol in liver microsomes from male and female rats. *J Biol Chem* **248:** 6559-6567, 1973.

Blizard D and Denef C, Neonatal androgen effects on open-field activity and sexual behavior in the female rat: the modifying influence of ovarian secretions during development. *Physiol Behav* **11:** 65-69, 1973.

Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248-254, 1976.

Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J and Guillemin R, Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* **179:** 77-79, 1973.

Cheng K-C and Schenkman JB, Purification and characterization of two constitutive forms of rat liver microsomal cytochrome P450. *J Biol Chem* **257:** 2378-2385, 1982.

Cheng K-C and Schenkman JB, Testosterone metabolism by cytochrome P450 isozymes RLM3 and RLM5 and by microsomes. *J Biol Chem* **258:** 11738-11744, 1983.

Cresteil T, Beaune P, Celier C, Leroux JP and Guengerich FP, Cytochrome P450 isoenzyme content and monooxygenase activities in rat liver: effect of ontogenesis and pretreatment by phenobarbital and 3-methylcholanthrene. *J Pharmacol Exp Therap* **236:** 269-276, 1986.

Dana-Haeri J, Oxley J and Richens A, Reduction of free testosterone by antiepileptic drugs. *Br Med J* **284:** 85-86, 1982.

Dannan GA, Guengerich FP and Waxman DJ, Hormonal regulation of rat liver microsomal enzymes. *J Biol Chem* **261:** 10728-10735, 1986.

De Moor P and Denef C, The "puberty" of the rat liver. Feminine pattern of cortisol metabolism in male rats castrated at birth. *Endocrinology* **82**: 480-492, 1968a.

Denef C, Effect of hypophysectomy and pituitary implants at puberty on the sexual differentiation of testosterone metabolism in rat liver. *Endocrinology* **94**: 1577-1582, 1974.

Denef C and De Moor P, The "puberty" of the rat liver. II. Permanent changes in steroid metabolizing enzymes after treatment with a single injection of testosterone propionate at birth. *Endocrinology* **83**: 791-798, 1968b.

Denef C and De Moor P, Sexual differentiation of steroid metabolizing enzymes in the rat liver. Further studies on predetermination by testosterone at birth. *Endocrinology* **91**: 374-384, 1972.

Dohler KD and Wuttke W, Changes with age in levels of serum gonadotropins, prolactin and gonadal steroids in prepubertal male and female rats. *Endocrinology* **97**: 898-907, 1975.

Edén S, Age- and sex-related differences in episodic growth hormone secretion in the rat. *Endocrinology* **105**: 555-560, 1979.

Einarsson K, Gustafsson J-A and Stenberg A, Neonatal imprinting of liver microsomal hydroxylation and reduction of steroids. *J Biol Chem* **248**: 4987-4997, 1973.

Elshourbagy NA and Guzelian PS, Separation, purification and characterization of a novel form of hepatic cytochrome P450 from rats treated with pregnenolone-16α-carbonitrile. *J Biol Chem* **255**: 1279-1285, 1980.

Elshourbagy NA, Barwich JL and Guzelian PS, Induction of cytochrome P450 by pregnenolone-16α-carbonitrile in primary monolayer cultures of adult rat hepatocytes and in a cell-free translation system. *J Biol Chem* **256**: 6060-6068, 1981.

Ewing GW, *Instrumental Methods of Chemical Analysis, 5th ed.* McGraw-Hill Book Company, New York, 1985, p 481.

Farris EJ, *The Care and Breeding of Laboratory Animals.* John Wiley & Sons, Inc, New York, 1950, p 43-78.

Fox JG, Cohen BJ, and Loew FM, *Laboratory Animal Medicine*. Academic Press, Inc, Orlando, 1984, p 91-123.

Finnen MJ and Hassall KA, Neonatal imprinting of sex differences in the hepatic metabolism of a chlorinated cyclodiene by rat liver microsomes. *Drug Metab Dispos* **12**: 127-130, 1984.

Forchielli E and Dorfman RI, Separation of Δ4-5α- and Δ4-5β-hydrogenases from rat liver homogenates. *J Biol Chem* **223**: 443-448, 1956.

Forest MG, Plasma androgens (testosterone and 4-androstenedione) and 17-hydroxyprogesterone in the neonatal, prepubertal and peripubertal periods in the human and the rat: differences between species. *J Steroid Biochem* **11**: 543-548, 1979.

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

Giachelli CM and Omiecinski CJ, Developmental regulation of cytochrome P450 genes in the rat. *Molec Pharmacol* **31**: 477-484, 1987.

Gilman AG, Goodman LS, Gilman A, *The Pharmacological Basis of Therapeutics, 6th ed.* MacMillan Publishing Co., New York, 1980, 1448-1461.

Giudicelli JF and Tillement JP, Influence of sex on drug kinetics in man. *Clin Pharmacokinetics* **2**: 157-166, 1977.

Gonzalez FJ, Song B-J and Hardwick JP. Pregnenolone-16α-carbonitrile-inducible P450 gene family: gene conversion and differential regulation. *Molec Cell Biol* **6**: 2969-2976, 1986.

Gonzalez FJ, Matsunaga T, Nagata K, Meyer UA, Nebert DW, Pastewka J, Kozak CA, Gillette J, Gelboin HV and Hardwick JP, Debrisoquine 4-hydroxylase: characterization of a new P450 gene subfamily, regulation, chromosomal mapping, and molecular analysis of the DA rat polymorphism. *DNA* **6**: 149-161, 1987.

Gonzalez FJ, The molecular biology of cytochrome P450s. *Pharmacol Rev* **40**: 243-288, 1989.

Gorski RA, Gonadal hormones and the perinatal development of neuroendocrine function. In: *Frontiers in Neuroendocrinology, 1971* (eds Martini L and Ganong WF), pp. 237-290. Oxford University Press, New York, 1971.

Goy RW and McEwen BS, *Sexual Differentiation of the Brain.* The MIT Press, Cambridge, Massachusetts, 1980, p 1-54.

Gozukara EM, Fagan J, Pastewka JV, Guengerich FP and Gelboin HV, Induction of cytochrome P450 mRNAs quantitated by *in vitro* translation and immunoprecipitation. *Arch Biochem Biophys* **232**: 660-669, 1984.

Graves PE, Kaminsky LS and Halpert, Evidence for functional and structural multiplicity of pregnenolone-16a-carbonitrile inducible cytochrome P450 isozymes in rat liver microsomes. *Biochemistry* **26**: 3887-3894, 1987.

Guengerich FP, Dannan GA, Wright ST, Martin MV and Kaminsky LS, Purification and characterization of liver microsomal cytochromes P450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**: 6019-6030, 1982.

Guengerich FP, *Mammalian Cytochromes P450, Volume I.* CRC Press, Inc., Boca Raton, Florida, 1987, p 1-54, 133-186.

Gustafsson J-A and Ingelman-Sundberg M, Regulation and substrate specificity of a steroid sulfate-specific hydroxylase system in female rat liver microsomes. *J Biol Chem* **250**: 3451-3458, 1975.

Gustafsson J-A and Stenberg A, Irreversible Androgenic programming at birth of microsomal and soluble rat liver enzymes on 4-androstene-3,17-dione and 5a-androstane-3a,17β-diol. *J Biol Chem* **249**: 711-718, 1974a.

Gustafsson J-A and Stenberg A, Neonatal programming of androgen responsiveness in liver of adult rats. *J Biol Chem* **249**: 719-723, 1974b.

Gustafsson J-A, Pousette A, Stenberg A and Wrange O, High-affinity binding of 4-androstene-3,17-dione in rat liver. *Biochemistry* **14**: 3942-3948, 1975.

Gustafsson J-A, Mode A, Norstedt G and Skett P, Sex steroid induced changes in hepatic enzymes. *Ann Rev Physiol* **45:** 51-60, 1983.

Halpert JR. Multiplicity of steroid-inducible cytochromes P450 in rat liver microsomes. *Arch Biochem Biophys* **263:** 59-68, 1988.

Harkness JE and Wagner JE. *The Biology and Medicine of Rabbits and Rodents, 2nd ed.* Lea & Febiger, Philadelphia, 1983, p43-51.

Hostetler KA, Wrighton SA, Kremers P and Guzelian PS, Immunochemical evidence for multiple steroid-inducible hepatic cytochromes P450 in the rat. *Biochem J* **245:** 27-33, 1987.

Imaoka S, Terano Y and Funae Y, Constitutive testosterone 6β-hydroxylase in rat liver. *J Biochem* **104:** 481-487, 1988.

Jacobson M and Kuntzman R, Studies on testosterone hydroxylation and pentobarbital oxidation by rat liver microsomes: selective effects of age, sex, castration and testosterone propionate treatment. *Steroids* **13:** 327-341, 1969.

Jansson J-O, Influence of neonatal and adult androgen secretion pattern of growth hormone in the rat. *Acta Endocrinology Supp* **256:** 163, 1983.

Jansson J-O, Ekberg S, Isaksson OGP and Edén S, Influence of gonadal steroids on age- and sex-related secretory patterns of growth hormone in the rat. *Endocrinology* **114:** 1287-1294, 1984.

Jansson J-O, Ekberg S, Isaksson O, Mode A and Gustafsson J-A, Imprinting of growth hormone secreton, body growth, and hepatic steroid metabolism by neonatal testosterone. *Endocrinology* **117:** 1881-1889, 1985a.

Jansson J-O, Edén and Isaksson O, Sexual dimorphism in the control of growth hormone secretion. *Endocrine Rev* **6:** 128-150, 1985b.

Jansson J-O, Mole J and Schenkman JB, Purification and characterization of a new form (RLM2) of liver microsomal cytochrome P450 from untreated rat. *J Biol Chem* **260:** 7084-7093, 1985c.

Jansson J-O and Frohman LA, Inhibitory effect of the ovaries on neonatal androgen imprinting of growth hormone secretion in female rats. *Endocrinology* **121**: 1417-1423, 1987.

Kalva PS and Kalra SP, Circadian periodicities of serum androgens, progesterone, gonadotropins and LH-RH in male rats: the effects of hypothalamic deafferentation, castration and adrenalectomy. *Endocrinology* **101**: 1821-1827, 1977.

Kamataki T, Maeda K, Yamazoe Y, Nagai T and Kato R, Sex differences of cytochrome P450 in the rat: purification, characterization, and quantitation of constitutive forms of cytochrome P450 from liver microsomes of male and female rats. *Arch Biochem Biophys* **225**: 758-770, 1983.

Kamataki T, Maeda K, Shimada M, Nagai T and Kato R. Neonatal testosterone imprinting of hepatic microsomal drug metabolism and a male-specific form of cytochrome P450 in the rat. *J Biochem* **96**: 1939-1942, 1984.

Kamataki T, Shimada M, Maeda K and Kato R, Pituitary regulation of sex-specific forms of cytochrome P450 in liver microsomes of rats. *Biochem Biophys Res Comm* **130**: 1247-1253, 1985.

Kato R and Kamataki T, Cytochrome P450 as a determinant of sex difference of drug metabolism in the rat. *Xenobiotica* **12**: 787-800, 1982.

Kato R, Yamazoe Y, Shimada M, Murayama N and Kamataki T, Effect of growth hormone and ectopic transplantation of pituitary gland on sex-specific forms of cytochrome P450 and testosterone and drug oxidations in rat liver. *J Biochem* **100**: 895-902, 1986.

Kato R, Sex-related difference in enzyme induction. In: *Enzyme Induction in Man* (Sotaniemi EA and Pelkonen RO, eds). pp 47-59. Taylor & Francis, London, 1987.

Kelly PA, Posner BI, Tsushima T and Friesen HG, Studies of insulin, growth hormone and prolactin binding: ontogenesis, effects of sex and pregnancy. *Endocrinology* **95**: 532-539, 1974.

Kinson GA and Liu C, Diurnal variation in plasma testosterone of the male laboratory rat. *Horm Metab Res* **5**: 233-234, 1973.

Kitada M, Kamataki T, Itahashi K, Rikihisa T and Kanakubo Y, P450 HFLa, a form of cytochrome P450 purified from human fetal livers, is the 16*a*-hydroxylase of dehydroepiandrosterone 3-sulfate. *J Biol Chem* **262:** 13534-13537, 1987.

Kitada M, Kamataki T, Itahashi K, Rikihisa T and Kanakubo Y, Significance of cytochrome P450 (P450 HLFa) of human fetal livers in the steroid and drug oxidations. *Biochem Pharmacol* **36:** 453-456, 1987.

Kitada M, Igoshi N, Kamataki T, Itahashi K, Imaoka S, Komori M, Funae Y, Rikihisa T and Kanakubo Y, Immunochemical similarity of P450 HFLa, a form of cytochrome P450 in human fetal livers, to a form of rat liver cytochrome P450 inducible by macrolide antibiotics. *Arch Biochem Biophys* **264:** 61-66, 1988.

Kleeberg U and Klinger W, Sensitive formaldehyde determination with Nash's reagent and a 'tryptophan' reaction. *J Pharmacol Methods* **8:** 19-31, 1982.

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

Kramer RE, Greiner JW, Rumbaugh RC, Sweeney TD and Colby HD, Requirement of the pituitary gland for gonadal hormone effects on hepatic drug metabolism in rats. *J Pharmacol Exp Ther* **208:** 19-23, 1979.

Kremers P, Beaune P, Cresteil T, De Graeve J, Columelli S, Leroux P and Gielen JE, Cytochrome P450 monooxygenase activities in human and rat liver microsomes. *J Biochem* **118:**599-606, 1981.

Larrey D, Distlerath LM, Dannan GA, Wilkinson GR and Guengerich FP, Purification and characterization of the rat liver microsomal cytochrome P450 involved in the 4-hydroxylation of debrisoquine, a prototype for genetic variation in oxidative drug metabolism. *Biochemistry* **4:** 2787-2795, 1984.

Leatherbarrow RJ, *Enzfitter. A Non-linear Regression Data Analysis Program for the IBM PC.* Elsevier-Biosoft, Cambridge, 1987.

Lee DW and Park KH, Testosterone metabolism by microsomal cytochrome P450 in liver of rats treated with some inducers. *Int J Biochem* **21:** 49-57, 1989.

Lemoine A, Marie S and Cresteil T, Expression of cytochrome P450 isozymes in the liver of hypophysectomized rats: evidence for different regulation mechanisms concerning P450IIB and P450IIIA subfamilies. *Eur J Biochem* **177**: 597-604, 1988.

Lisboa BP and Gustafsson J-A, Biosynthesis of two new steroids in the human foetal liver, 1β- and 2β-hydroxytestosterone. *J Biochem* 6:419-424, 1968.

Lorenz K, *Studies in Animal and Human Behavior.* Methuen, London, 1971, p124-129. Originally published as Uber Tierisches und menschliches Verhalten. R. Piper, Munchen, 1965.

MacAdams MR, White RH and Chipps BE, Reduction of serum testosterone levels during chronic glucocorticoid therapy. *Ann Int Med* **104**:648-651, 1986.

MacGeoch C, Morgan ET, Halpert J and Gustafsson J-A, Purification, characterization, and pituitary regulation of the sex-specific cytochrome P450 15β-hydroxylase from liver microsomes of untreated female rats. *J Biol Chem* **259**: 15433-15439, 1984.

MacGeoch C, Morgan ET, Cordell B and Gustafsson J-A, Growth hormone regulates expression of rat liver cytochrome P45015β at a pretranslational level, *Biochem Biophys Res Commun* **143**: 782-788, 1987.

MacLusky NJ and Naftolin F, Sexual differentiation of the central nervous system. *Science* **211**: 1294-1311, 1981.

Massart DL, Dijkstra A and Kaufman L, *Evaluation and Optimization of Laboratory Methods and Analytical Procedures.* Elsevier Scientific Publishing Co., Amsterdam, 1978, p 143-156.

McClellan-Green P, Linko P and Goldstein JA, Developmental regulation of male specific cytochrome P450g in livers of male and female ACI rats. *FASEB J* **3**: 2085, 1989.

McDonald PG and Doughty C, Inhibition of androgen-sterilization in the female rat by administration of an antioestrogen. *J Endocr* **55**: 455-456, 1972.

Meffin PJ, Brooks PM and Sallustio BC, Alterations in prednisolone disposition as a result of time of administration, gender and dose. *Br J Clin Pharmac* **17**:395-404, 1984.

Mock EJ, Norton HW and Frankel AI, Daily rhythmicity of serum testosterone concentration in the male laboratory rat. *Endocrinology* **103**: 1111-1121, 1978.

Mode A, Gustafsson J-A, Jansson J-O, Edén S and Isaksson O, Association between plasma level of growth hormone and sex differentiation of hepatic steroid metabolism in the rat. *Endocrinology* **111**:1692-1697, 1982.

Mode A, Wiersma-Larsson E, Strom A, Zaphiropoulos PG and Gustafsson J-A, A dual role of growth hormone as a feminizing and masculinizing factor in the control of sex-specific cytochrome P450 isozymes in rat liver. *J Endrocr* **120**: 311-317, 1988.

Morgan ET, MacGeoch C and Gustafsson J-A, Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16a-hydroxylase cytochrome P450 apoprotein in the rat. *J Biol Chem* **260**: 11895-11898, 1985.

Naftolin F, Ryan KJ, Davies IJ, Reddy VV, Flores F, Petro Z and Kuhn M, The formation of estrogens by central neuroendocrine tissues. *Recent Prog Horm Res* **31**: 295-315, 1975.

Nagata K, Matsunaga T, Gillette J, Gelboin HV and Gonzalez FJ, Rat testosterone 7a-hydroxylase: Isolation, sequence, and expression of cDNA and its developmental regulation and induction by 3-methylcholanthrene. *J Biol Chem* **262**: 2787-2793, 1987.

Nash T, The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* **55**: 416-421, 1953.

Nebert DW, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levinin W, Phillips IR, Sato R and Waterman MR, The P450 gene superfamily: recommended nomenclature. *DNA* **6**: 1-11, 1987.

Nebert DW, Nelson DR, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Kemper B, Levinin W, Phillips IR, Sato R and Waterman MR, The P450 Superfamily: updated listing of all genes and recommended nomenclature for the chromosomal loci. *DNA* **8**: 1-13, 1989.

Norusis MJ, *SPSS-X Introductory Statistics Guide*. SPSS Inc., Chicago, 1988, p 121.

Ochs HR, Greenblatt DJ, Divoll M, Abernethy DR, Feyerabend H and Dengler HJ, Diazepam kinetics in relation to age and sex. *Pharmacology* **23**: 24-30, 1981.

Ohgiya N, Yokota H and Yuasa A, Purification and properties of cytochrome P450 generally acting as a catalyst on benzo[a]pyrene hydroxylation from liver microsomes of untreated rats. *J Biochem* **105**: 234-238, 1989.

Omura T and Sato R, A new cytochrome in liver microsomes. *J Biol Chem* **237**: PC1375-PC1376, 1962.

Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370-2378, 1964a.

Omura T and Kato R, The carbon-monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties. *J Biol Chem* **239**: 2379-2385, 1964b.

Pak RCK, Tsim KWK and Cheng CHK, Pubertal gonadal hormones in modulating the testosterone dependency of hepatic aryl hydrocarbon hydroxylase in female rats. *Pharmacology* **29**: 121-127, 1984.

Parkinson A, Thomas PE, Ryan DE, Reik LM, Safe SH, Robertson LW and Levinin W, Differential time course of induction of rat liver microsomal cytochrome P450 isozymes and epoxide hydrolase by Aroclor 1254. *Arch Biochem Biophys* **225**: 203-215, 1983.

Pfaffenberger CD and Horning EC, Sex profiles in human urinary steroid metabolic profiles determined by gas chromatography. *Analyt Biochem* **80**: 329-343, 1977.

Proksch RA and Lamy PP, Sex variation and drug therapy. *Drug Intell Clin Pharm* **11**:398-406, 1977.

Raynaud J-P, Mercier-Bodard C and Baulieu EE, Rat estradiol binding plasma protein (EBP). *Steroids* **18**: 767-788, 1971.

Rieutort M, Pituitary content and plasma levels of growth hormone in foetal and weanling rats. *J Endocr* **60**: 261-268, 1974.

Roberts RK, Desmond PV, Wilkinson GR and Schenker S, Disposition of chlordiazepoxide: sex differences and effects of oral contraceptives. *Clin Pharm Ther* **25**: 826-831, 1979.

Roy AK and Chatterjee B, Sexual dimorphism in the liver. *Ann Rev Physiol* **45**: 37-50, 1983.

Roy AK, Chatterjee B, Demyan WF, Milin BS, Motwani NM, Nath S and Schiop MJ, Hormone and age-dependent regulation of a2u-globulin gene expression. *Recent Prog Horm Res* **39**: 425-457, 1983.

Ryan DE, Thomas PE, Korzeniowski D and Levinin W, Separation and characterization of highly purified forms of liver microsomal cytochrome P450 from rats treated with polychlorinated biphenyls, phenobarbital, and 3-methylcholanthrene. *J Biol Chem* **254**: 1365-1374, 1979.

Ryan DE, Thomas PE and Levinin W, Hepatic microsomal cytochrome P450 from rats treated with isosafrole. *J Biol Chem* **255**: 7941-7955, 1980.

Ryan DE, Thomas PE and Levinin W, Purification and characterization of a minor form of hepatic microsomal cytochrome P450 from rats treated with polychlorinated biphenyls. *Arch Biochem Biophys* **216**: 272-288, 1982.

Ryan DE, Iida S, Wood AW, Thomas PE, Lieber CS and Levinin W, Characterization of three highly purified cytochromes P450 from hepatic microsomes of adult male rats. *J Biol Chem* **259**: 1239-1250, 1984.

Ryan DE, Ramanathan L, Iida S, Thomas PE, Haniu M, Shively JE, Lieber CS and Levinin W, Characterization of a major form of rat hepatic microsomal cytochrome P450 induced by isoniazid. *J Biol Chem* **260**: 6385-6393, 1985.

Shimada M, Murayama N, Yamazoe Y, Kamataki T and Kato R, Further studies on the persistence of neonatal androgen imprinting on sex-specific cytochrome P450, testosterone and drug oxidations. *Japan J Pharmacol* **45**: 467-478, 1987.

Shimada M, Murayama N, Yamauchi K, Yamazoe Y and Kato R, Suppression in the expression of a male-specific cytochrome P450, P450-male: difference in the effect of chemical inducers on P450-male mRNA and protein in rat livers. *Arch Biochem Biophys* **270**: 578-587, 1988.

Skett P and Gustafsson J-A, Imprinting of enzyme systems of xenobiotic and steroid metabolism. In: *Reviews in Biochemical Toxicology* (Eds. Hodgson E, Bend JR and Philpot RM), pp. 27-52. Elsevier North Holland, Inc., New York, 1979.

Sloop TC, Clark JC, Rumbaugh RC and Lucier GW, Imprinting of hepatic estrogen-binding proteins by neonatal androgens. *Endocrinology* **112**: 1639-1646, 1983.

Smith RV and Stewart JT, *Textbook of Biopharmaceutic Analysis.* Lea & Febiger, Philadelpphia, 1981, p 277.

Sonderfan AJ, Arlotto MP, Dutton DR, McMillen SK and Parkinson A, Regulation of testosterone hydroxylation by rat liver microsomal cytochrome P450. *Arch Biochem Biophys* **255**: 27-41, 1987.

Song B-J, Gelboin HV, Park S-S, Yang CS and Gonzalez FJ, Complementary DNA and protein sequences of ethanol-inducible rat and human cytochrome P450s. *J Biol Chem* **261**: 16689-16697, 1986.

Stedman's Medical Dictionary, 23 ed., The William & Wilkins Company, Baltimore, 1976.

Stenberg A, On the modulating effects of ovaries on neonatal androgen programming of rat liver enzymes. *Acta Endocrinol* **78**: 294-301, 1975.

Strom A, Mode A, Morgan E and Gustafsson J-A, Pretranslational hormonal control of male-specific cytochrome P45016a in rat liver. *Biochem Soc Trans* **15**: 575-576, 1987.

Swinney DC, Ryan DE, Thomas PE and Levinin W, Regioselective progesterone hydroxylation catalyzed by eleven rat hepatic cytochrome P450 isozymes. *Biochemistry* **26**: 7073-7083, 1987.

Thomas PE, Reik LM, Ryan DE and Levinin W, Regulation of three forms of cytochrome P450 and epoxide hydrolase in rat liver microsomes. *J Biol Chem* **256**: 1044-1052, 1981.

Thomas PE, Bandiera S, Maines SL, Ryan DE and Levinin W, Regulation of cytochrome P450j, a high-affinity N-nitrosodimethylamine demethylase, in rat hepatic microsomes. *Biochemistry* **26**: 2280-2289.

Thrasher K, Werk EE, Choi Y, Sholiton LJ, Meyer W and Olinger C, The measurement, excretion and source of urinary 6-hydroxycortisol in humans. *Steroids* **14**:455-463, 1969.

Uriel J, de Nechaud B and Dupiers M, Estrogen-binding properties of rat, mouse and man fetospecific serum proteins. Demonstration by immuno-autoradiographic methods. *Biochem Biophys Res Comm* **46**: 1175-1180, 1972.

Walle T, Walle UK, Cowart TD and Conradi EC, Pathway-selective sex differences in the metabolic clearance of propranolol in human subjects. *Clin Pharmacol Ther* **46**:257-263, 1989.

Watkins PB, Wrighton SA, Schuetz EG, Maurel P and Guzelian PS, Macrolide antibiotics inhibit the degradation of the glucocorticoid-responsive cytochrome P450p in rat hepatocytes *in vivo* and in primary monolayer culture. *J Biol Chem* **261**: 6264-6271, 1986.

Waxman DJ and Walsh C, Cytochrome P450 isozyme 1 from phenobarbital-induced rat liver: purification, characterization and interactions with metyrapone and cytochrome b5. *Biochemistry* **22**: 4846-4855, 1983.

Waxman DJ, Ko A and Walsh C, Regioselectivity and stereoselectivity of androgen hydroxylations catalyzed by cytochrome P450 isozymes purified from phenobarbital-induced rat liver. *J Biol Chem* **258**: 11937-11947, 1983.

Waxman DJ, Rat hepatic cytochrome P450 isoenzyme 2c: identification as a male-specific, developmentally induced steroid 16a-hydroxylase and comparison to a female-specific cytochrome P450 isoenzyme. *J Biol Chem* **259**: 15481-15490, 1984.

Waxman DJ, Dannan GA and Guengerich FP, Regulation of rat hepatic cytochrome P450: age-dependent expression, hormonal imprinting, and xenobiotic inducibility of sex-specific isoenzymes. *Biochemistry* **24**: 4409-4417, 1985.

Waxman DJ, LeBlanc GA, Morrissey JJ, Staunton J and Lapenson DP, Adult male-specific and neonatally programmed rat hepatic P450 forms RLM2 and 2a are not dependent on pulsatile plasma growth hormone for expression. *J Biol Chem* **263**: 11396-11406, 1986.

Weisz J and Ward IL, Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. *Endocrinology* **106**: 306-316, 1980.

Wiebel FJ and Gelboin HV, Aryl hydrocarbon (benzo[a]pyrene) hydroxylases in liver from rats of different age, sex and nutritional status. *Biochem Pharmacol* **24**: 1511-1515, 1975.

Williams MT and Simonet LC, Effects of growth hormone on cytochrome P450j. *Biochem Biophys Res Commun* **155**:392-397, 1988.

Wood AW, Ryan DE, Thomas PE and Levinin W, Regio- and stereoselective metabolism of two C_{19} steroids by five highly purified and reconstituted rat hepatic cytochrome P450 isozymes. *J Biol Chem* **258**: 8839-8847, 1983.

Wrange A, Norstedt G and Gustafsson J-A, The estrogen receptor in rat liver: quantitative and qualitative analysis by isoelectric focusing in polyacrylamide gel. *Endocrinology* **106**: 1455-1463, 1980.

Wrighton SA, Maurel P, Schuetz EG, Watkins PB, Young B and Guzelian PS, Identification of the cytochrome P450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P450p. *Biochemistry* **24**: 2171-2178, 1985a.

Wrighton SA, Schuetz EG, Watkins PB, Maurel P, Barwick J, Bailey J, Bailey BS, Hartle HT, Young B and Guzelian P, Demonstration in multiple species of inducible hepatic cytochromes P450 and their mRNAs related to the glucocorticoid-inducible cytochrome P450 of the rat. *Molec Pharmacol* **28**: 312-321, 1985b.

Yamazoe Y, Murayama N, Shimada M, Yamauchi K, Nagata K, Imaoka S, Funae Y and Kato R, A sex-specific form of cytochrome P450 catalyzing propoxycoumarin O-depropylation and its identity with testosterone 6β-hydroxylase in untreated rat livers: reconstitution of the activity with microsomal lipids. *J Biochem* **104**: 787-790, 1988.

Yates FE, Herbst AL and Urquhart J, Sex difference in rate of ring reduction of Δ4-3-keto-steroids *in vitro* by rat liver. *Endocrinology* **63**: 887-902, 1958.

Zaphiropoulos PG, Mode A, Norstedt G and Gustafsson J-A, Regulation of sexual differentiation in drug and steroid metabolism. *Trends in Pharmacol Sci* **10**: 149-153, 1989.

6. APPENDIX

Table A. Pilot study: results in ovariectomized female rats. From data obtained by RCK Pak (liver and body weights) and S. Bandiera (P450). Results are presented as mean ± S.E.M.. and are also contained in Table B. Abbreviations: Fem, Female; Gx, prepubertal gonadectomy; Ta, adult testosterone treatment; Tp, pubertal testosterone treatment; "*" indicates a significant difference from each other in that row (p<0.05); "**" indicate a significant difference from each other in that row (p<0.05).

Table A.

	TREATMENT GROUP			
	Fem Gx	Fem GxTa	Fem GxTp	Fem GxTpTa
mg protein per gram wet weight liver	48.5±3.9	57.9±4.6	55.8±6.4	50.1±2.9
nmoles P450 per gram wet weight liver	41.6±5.9	47.5±4.7	48.2±4.3	44.1±7.0
nmoles P450 per mg protein in hepatic microsomes	0.85±0.05	0.83±0.02	0.87±0.03	0.87±0.09
Body Weight (g)	* 351±9	315±8	* 300±11	336±13
Liver Weight (g)	10.5±0.7	9.2±0.4	9.2±0.7	11.6±0.7
Liver/Body (%)	3.0±0.1	2.9±0.1	** 3.0±0.1	** 3.4±0.1

Table B. Pilot study: results in all treatment groups. From data obtained by RCK Pak (liver and body weights) and S. Bandiera (P450). Dta is presented as mean ± S.E.M.. and is also contained in Table B. Abbreviations: Fem, Female; Gx, prepubertal gonadectomy; Ta, adult testosterone treatment; Tp, pubertal testosterone treatment; *, for statistics refer to Table C; "**", indicates a significant difference from Con Fem, Fem GxTa, Fem GxTp, Fem GxEp and Fem GxEpTa groups in that row (p<0.05); "***" indicates a significant difference from Fem Gx, Fem GxTa and Fem GxTp groups in that row (p<0.05).

Table B.

	CON Male	CON Fem	Fem Gx	TREATMENT GROUPS Fem GxTa	Fem GxTp	Fem GxTpTa	Fem GxEp	Fem GxEpTa
nmoles P450 per mg protein	0.97±0.10	0.82±0.02	0.85±0.05	0.82±0.02	0.87±0.03	0.87±0.09	0.87±0.09	0.79±0.03
nmoles P450 per g wet weight of liver	42±1	52±2	42±6	48±5	48±4	44±7	51±5	45±2
mg protein per g wet weight of liver	44±4	64±4	49±4	58±5	56±6	50±3	59±3	57±3
Body Weight (g) *	517±18	240±13	351±9	315±8	300±11	336±13	233±9	225±8
Liver Weight(g)	18.2±0.7	8.2±0.6	10.5±0.7	9.2±0.4	9.2±0.7	** 11.6±0.7	8.4±0.4	8.7±0.6
Liver/Body (%)	3.51±0.04	3.37±0.07	2.99±0.14	2.93±0.11	3.04±0.13	3.44±0.13	*** 3.62±0.04	*** 3.87±0.21

Table C. Pilot study: statistics for body weights. "*"

indicates a significant difference between groups (p<0.05).

Treatment groups:
Grp 2: Con Fem
Grp 3: Fem Gx
Grp 4: Fem GxTa
Grp 5: Fem GxTp
Grp 6: Fem GxTpTa
Grp 7: Fem GxEp
Grp 8: Fem GxEpTa

		G	G	G	G	G	G	G
		r	r	r	r	r	r	r
		p	p	p	p	p	p	p
Mean	Group	8	7	2	5	4	6	3
225.0000	Grp 8							
233.0000	Grp 7							
239.5000	Grp 2							
299.5000	Grp 5			*	*	*		
315.0000	Grp 4			*	*	*		
336.0000	Grp 6			*	*	*		
351.0000	Grp 3			*	*	*	*	

Table D. Major study: statistics for transformed erythromycin demethylase (natural logarithm of nmoles formaldehyde per minute per mg protein). "*" indicates a significant difference between groups ($p<0.05$). Treatment groups:
Grp 1: Con Fem
Grp 2: Con Fem Ta
Grp 3: Fem Gx
Grp 4: Fem GxTa
Grp 5: Fem GxTp
Grp 6: Fem GxTpTa
Grp 7: Con Male
Grp 8: Con Male Ta
Grp 9: Male Gx
Grp 10: Male GxTa
Grp 11: Male GxTp
Grp 12: Male GxTpTa

Mean	Group	Grp3	Grp9	Grp4	Grp5	Grp2	Grp10	Grp1	Grp11	Grp6	Grp12	Grp7	Grp8
-1.1905	Grp 3												
-1.1202	Grp 9												
-1.1178	Grp 4												
-1.1075	Grp 5												
-.9305	Grp 2												
-.7700	Grp10												
-.7320	Grp 1	*											
-.7311	Grp11												
-.6167	Grp 6	*	*	*	*								
-.3223	Grp12	*	*	*	*	*			*	*			
-.3145	Grp 7	*	*	*	*	*			*	*			
.1325	Grp 8	*	*	*	*	*	*	*	*	*	*	*	

Table E. Major study: statistics for transformed erythromycin demethylase (natural logarithm of nmoles formaldehyde per minute per nmole P450). "*" indicates a significant difference between groups ($p < 0.05$). Treatment groups:

Grp 1: Con Fem
Grp 2: Con Fem Ta
Grp 3: Fem Gx
Grp 4: Fem GxTa
Grp 5: Fem GxTp
Grp 6: Fem GxTpTa
Grp 7: Con Male
Grp 8: Con Male Ta
Grp 9: Male Gx
Grp 10: Male GxTa
Grp 11: Male GxTp
Grp 12: Male GxTpTa

Mean	Group	Grp3	Grp9	Grp4	Grp5	Grp2	Grp11	Grp1	Grp10	Grp6	Grp12	Grp7	Grp8
-1.3577	Grp 3												
-1.1781	Grp 9												
-1.1741	Grp 4												
-1.1228	Grp 5												
-1.0383	Grp 2												
-.9074	Grp 11	*											
-.8469	Grp 1	*											
-.8213	Grp 10	*											
-.7833	Grp 6	*											
-.5757	Grp 12	*	*	*	*	*							
-.4884	Grp 7	*	*	*	*	*	*						
-.1872	Grp 8	*	*	*	*	*	*	*	*	*	*		

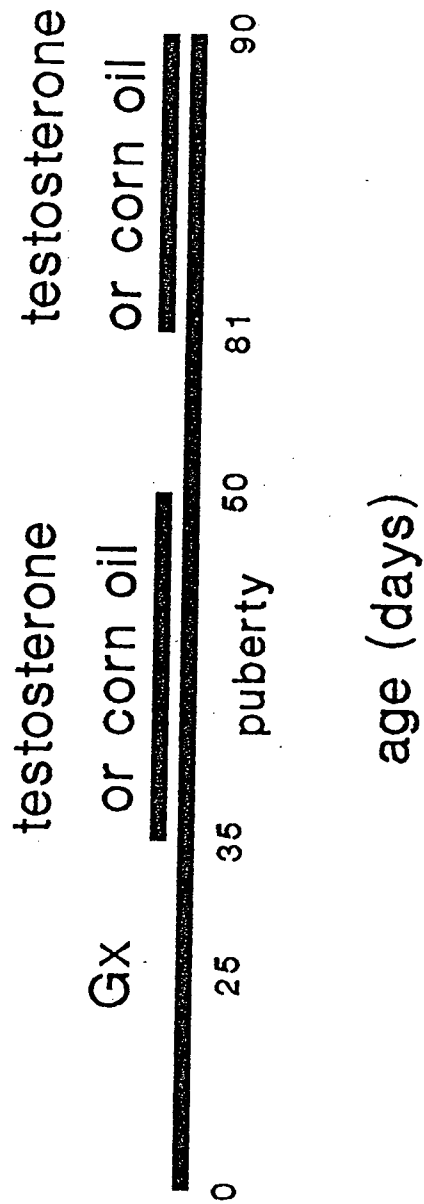Figure 1. Scheme of methodology used in the major study.

Figure 2. ANOVA table for data on gonadectomized female rats in the pilot study. Values represent the mean erythromycin demethylase activity per mg microsomal protein (upper table) or per nmole P450 (lower table).

```
                 TADULT
                        0            1
      TFUB
            0           .29          .33
                   (      4)   (      4)

            1           .35          .59
                   (      4)   (      4)
```

```
                 TADULT
                        0            1
      TFUB
            0           .36          .41
                   (      4)   (      4)

            1           .41          .74
                   (      4)   (      4)
```

Figure 3. ANOVA table for data on gonadectomized female rats in the major study. Values represent the mean erythromycin demethylase activity per mg protein (upper table) or per nmole P450 (lower table).

```
              TADULT
                    0              1
   TFUB
       0           .31           .34
              (      9)   (      9)

       1           .33           .55
              (      9)   (      9)
```

```
              TADULT
                    0              1
   TFUB
       0           .26           .32
              (      9)   (      9)

       1           .33           .47
              (      9)   (      9)
```