PERIPUBERTAL DEFEMINIZATION OF HEPATIC CYTOCHROMES P-450 2C11 AND 3A2 BY TESTICULAR STEROIDS IN THE ABSENCE OF NEONATAL DEFEMINIZATION

JENNIFER LEA AVERY

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by

Jennifer Lea Avery

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ABSTRACT

PERIPUBERTAL DEFEMINIZATION of HEPATIC CYTOCHROMES P-450 2C11 and 3A2 by TESTICULAR STEROIDS in the ABSENCE of NEONATAL DEFEMINIZATION

by Jennifer Lea Avery

We studied the ability of testicular secretions to either defeminize (represented as an increase in basal specific activity), or masculinize (represented as an ability to respond to adult testosterone administration), the sex-specific P-450s 2C11 and 3A2, in the absence of neonatal defeminization. Neonatal defeminization of these P-450s is suspected to occur *via* androgen-derived estrogens. Defeminization was successfully prevented by subcutaneous insertion of Silastic™ capsules containing the aromatase blocker 1,4,6-androstatriene-3,17-dione (ATD), from day 0 (birth) to day 21 (weaning), in the male rat. The testes remained *in situ* from birth to day 21, 35, 55, 70, or death. Animals castrated on day 21 or 55 received either testosterone therapy (2 mg/kg/day, s.c., in corn oil) for 14 days, beginning on day 70, or received no testosterone therapy. All animals were killed on day 70 or 84.

The neonatal ATD treatment blocked the normal increase in basal ethylmorphine demethylase (EMDM), 6β-hydroxylase, 2α-hydroxylase, and 16α-hydroxylase specific activity, seen in adulthood. Although ATD prevented the neonatal defeminization as noted by a permanent increase in basal activity, both P-450 isozymes were responsive to testosterone in adulthood, either partially (EMDM), or completely (6β-, 2α-, 16α-hydroxylase).

The intact testes from birth to death, in the ATD-treated male, effected a *permanent* increase in the basal activities of EMDM, as well as the 6β-, 2α-, and 16α-hydroxylations on testosterone. This treatment also effected a 100% recovery (*i.e.* intact male activity) of EMDM activity, when stimulated by testosterone in

adulthood. The permanent increase in EMDM basal activity, is most likely completed by day 55, as there was no difference in specific activity between the day 55 castrate, and the day 70 castrate. Of the hydroxylations of testosterone, we demonstrate that castration on day 55 results in significantly *higher* specific activities of these isozymes, then castration on day 70.

We also report significant sex differences (males > females) in aniline hydroxylase activity, cytochrome P-450 content, and cytochrome *c* reductase activity. We do not attribute the above results to differences in P-450 content or reductase activity. The 7α-hydroxylation of testosterone (P-450 2A1) was sexually differentiated (females > males). We demonstrated that the elimination of estrogens throughout the life of the male (ATD treated and castrated on day 21), prevented the feminization (increase in basal activity) of 7α-hydroxylase, following castration.

These results demonstrate that puberty is a very dynamic process, resulting in changing characteristics of these sex-specific P-450s. Puberty is also a time when these basal enzyme activities, as well as the responsiveness of EMDM to adult testosterone therapy, can be permanently defeminized, in the absence of neonatal defeminization.

...

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1.0. INTRODUCTION

Cytochrome P-450 denotes a super-family of heme-protein enzymes that have been described in 31 eukaryotes (including 11 mammals and 3 plants) and 11 prokaryotes. The name '*cytochrome P-450*' was actually given to a red pigment having, in the reduced carbon monoxide (CO)-difference spectrum, a major band at the wavelength of 450 nm (Omura and Sato, 1964). This ubiquitous enzyme family is responsible for a large number of metabolic processes, including the metabolism of foreign compounds (*xenobiotics*) as well as the metabolism of compounds inherent to the host (*i.e.* steroids). In mammals, the enzyme system has been found in all tissues examined.

Many P-450 isozymes[1] exist within a specific tissue, however each isozyme performs its own variety of metabolic reactions. Many isozymes may be responsible for one reaction, or one isozyme may be responsible for a number of different reactions. Cytochromes P-450 are often described as having "*broad and overlapping substrate specificities*".

Mammalian species host 12 families of P-450s, of which at least six families include P-450 forms involved in steroidogenesis (Gonzalez, 1988; Gonzalez and Nebert, 1990). The 12 mammalian families have 22 subfamilies, and these

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[1] any of a set of structural variants of an enzyme occurring in different tissues in a single species (from *The Collins English Dictionary*, 2nd edition)

subfamilies originate from clusters of tightly linked genes. Nelson *et al.* (1993) suggested a common nomenclature for naming individual P-450 isozymes. To demonstrate, examine the P-450 "*3A2*". The first Arabic numeral "3", denotes the P-450 *family*, the following letter "A", designates the *subfamily* (if two or more exist), and the Arabic number "2", represents the *individual gene product* within the subfamily. Genetic homology determines which isozyme belongs to which family and subfamily. By definition, a P-450 family will have a 40% homology in amino acids with all other P-450 proteins in the same family. Similarly, mammalian sequences within the same subfamily are > 55% identical (Nelson *et al.*, 1993). Past literature has presented the reader with a variety of nomenclature systems for identifying individual P-450 isozymes. For simplicity, we will follow the system proposed by Nelson *et al.* (1993) as defined above. For analogous names of P-450 isozymes given in different laboratories, see **Appendix B**.

Typical P-450 monooxygenase[2] reactions include hydroxylation, epoxidation, deamination and desulfuration, among many others (Sipes and Gandolfi, 1986). Generally, a P-450 reaction in a microsomal[3] system begins with the transfer of electrons from NAD(P)H to NADPH-cytochrome P-450 reductase and then to cytochrome P-450. This reduces the molecular oxygen species, and is followed by the insertion of one oxygen atom into the substrate (*oxidation*). However P-450s can also *reduce* substrates.

---

[2] an enzyme introducing one atom of oxygen from O_2 into a compound while reducing the other atom of oxygen to water

[3] any of the small particles consisting of ribosomes and fragments of attached endoplasmic reticulum that can be isolated from cells by centrifugal action

The following is the general stoichiometric formula for a typical P-450 oxygenation, with 'R' representing the substrate.

$$RH + O_2 + NADPH + H^+ \quad ROH + H_2O + NADP^+$$

Generally, the substrates are *lipo*philic (lipid soluble), and the oxygenation process ultimately converts the substrate to a more *hydro*philic (water soluble) state, so that the derivative is more readily excreted by either the kidneys or the biliary fecal route. This process is termed *biotransformation*. Unfortunately, some inert (*i.e.* non-toxic) parent compounds may be biotransformed by P-450s to secondary molecules, which are capable of causing toxic (*i.e.* mutagenic, carcinogenic) damage to body cells, tissues, or organs. This event is termed *bioactivation*. As well, some toxic parent compounds can be rendered biologically inert by P-450 metabolism, thus sparing the body tissues from damage (*detoxification*).

Along with the excretion of foreign compounds from the body, some of the P-450 reactions are essential for endogenous physiological processes. For example, a specific group of P-450s carry out the conversion of cholesterol to sex hormones. Following the hormonal action, P-450s may again be responsible for the ultimate deactivation and elimination of the hormone derivative.

1.1. Sex-specific hepatic P-450s

By definition, sex-specific P-450s are those which are present and expressed (active) in one sex, and may be present but *not* expressed (inactive) in the opposite sex. Of the hepatic cytochromes P-450 found in the rat, the expression of a number of these, characterized in terms of V_{max}[4] (maximum velocity), K_m[5] (Michaelis constant), and/or simply *specific activity* (nmol product formed per minute per milligram of microsomal protein), are sexually dimorphic. For example, the metabolism of the substrates ethylmorphine, hexobarbital, and aminopyrine, by rat hepatic microsomes, is dimorphically characterized, males exhibiting a significantly greater V_{max} and females exhibiting a significantly greater K_m (Castro and Gillette, 1967; Kato and Onoda, 1970; Chung, 1977; Reyes and Virgo, 1988).

In terms of specific activity (*i.e.* apparent V_{max}), the metabolism of the drug ethylmorphine hydrochloride, has been shown to occur 2.5 - 6 times faster in the male rat compared to the female (Castro and Gillette, 1967; El Defrawy El Masry *et al.*, 1974; Virgo, 1991). Similarly, the metabolism of both hexobarbital and aminopyrine are 2-3 times faster in the male as opposed to the female (Schenkman *et al.*, 1967; Kato *et al.*, 1968; MacLeod *et al.*, 1972). Although these differences have been found in various rat strains (*i.e.* Long-Evans, Holtzman, Wistar, Sprague-Dawley), the

---

[4] the theoretical limit for the rate of reaction under defined conditions when the substrate concentration is so high that the active site is constantly occupied by substrate

[5] the substrate concentration at which the actual velocity is 1/2 of the maximum velocity with no product present

same sex differences have not necessarily been found in other *species*, including mice (Kato *et al.*, 1968), rabbits (Castro and Gillette, 1967; Kato *et al.*, 1968), guinea pigs (Castro and Gillette, 1967), and monkeys (Castro and Gillette, 1967).

In the time since these initial discoveries, a large number of sex differences have been found in the metabolism of drugs, chemicals and hormones. For example, the sex-specific metabolism of testosterone, at various molecular positions, is catalyzed by a number of P-450 isozymes, with a high degree of regio- and stereoselectivity. Each specific metabolic pathway can be correlated with its respective P-450(s). The steroid hydroxylation reactions are named according to which carbon (*numeral*), ring (*letter*) and face (*α or β*), is involved in the reaction (Waxman, 1988).

2α-hydroxylation	=	α-face on the A-ring
16α-hydroxylation	=	α-face on the D-ring
6β-hydroxylation	=	β-face on the B-ring
7α-hydroxylation	=	α-face on the B-ring

We will study the metabolism of ethylmorphine hydrochloride (substrate) to formaldehyde (product), as well as the hydroxylation of testosterone at three positions, namely the 2α–, 16α-, and 6β–positions. We chose these specific metabolic reactions as P-450 markers because the major isozymes responsible for these reactions (P-450s 2C11 and 3A2) are specifically expressed in the adult *male* rat.

The 2α-hydroxylation of testosterone is metabolized exclusively (*i.e.* 100%) by P-450 **2C11**. For future reference, 2C11 will be considered *specific* for the metabolism of testosterone at the 2α position. In other words, the specific activity of 2α-hydroxylase will be the marker representing the amount of 2C11 present. The following is compiled from Wood *et al.* (1983), Ryan *et al.* (1984a), and Ryan *et al.* (1984b).

Approximately forty-two percent (42%) of testosterone 16α-hydroxylations are catalyzed by P-450 **2C11**, the balance of 16α-hydroxylations being catalyzed by other P-450 isozymes (**Figure 1.1.A.**). Thus, 2C11 is *selective*, but not specific, for the metabolism of testosterone at the 16α– position. The specific activity of 16α-hydroxylase will only be a partial indication of the amount of 2C11 present in the microsomes.

Approximately fifty-seven percent (57%) of testosterone 6β-hydroxylations are catalyzed by P-450 **3A2**, the balance of 6β-hydroxylations being catalyzed by other P-450 isozymes (**Figure 1.1.B.**). In the same context, **3A2** is only *selective* for the 6β-hydroxylation of testosterone (57%), but is *specific* for the metabolism of ethylmorphine. Thus, ethylmorphine demethylase (EMDM) activity will give a better indication of the amount of P-450 3A2 present.

We will also be measuring the specific activity of the isozyme responsible for the 7α-hydroxylation of testosterone, as this reaction is *female*-predominant, but not *specific* to the female (Sonderfan *et al.*, 1987; Waxman *et al.*, 1989). Since the 7α-hydroxylation of testosterone is performed in the most part, by P-450 **2A1**, and 7α-

hydroxylase activity has been shown to reflect levels of 2A1 (Sonderfan *et al.*, 1987), this will enable us to determine if and/or how hormonal manipulation in the male rat affects the activity of this isozyme.

We will also be determining the effects of hormonal manipulation on the metabolism of aniline (catalyzed by *aniline hydroxylase*), a reaction that literature suggests is not sexually differentiated in the adult rat (Schenkman *et al.*, 1967; MacLeod *et al.*, 1972; El Defrawy El Masry *et al.*, 1974). The activity of P-450 **2E1** will be correlated with aniline hydroxylase activity, as up to 60% of the oxidative metabolism of the aromatic aniline to form *p*-aminophenol, has been shown to be catalyzed by this P-450 isozyme (Koop and Coon, 1986; Ko *et al.*, 1987).

Figure 1.1.A. P-450 isozymes responsible for the 16α-hydroxylation of testosterone (Wood *et al.*, 1983; Ryan *et al.*, 1984a; Ryan *et al.*, 1984b).

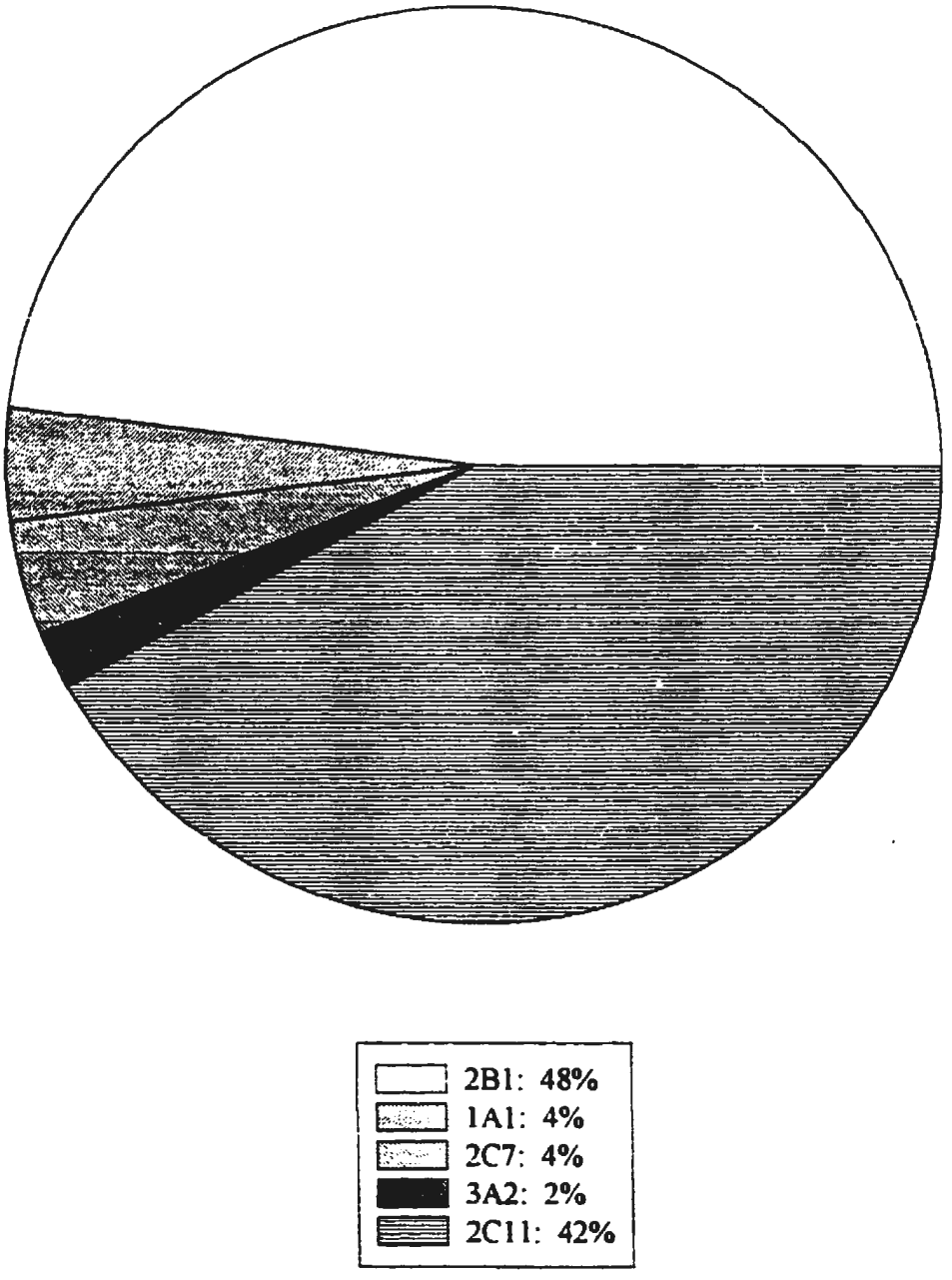
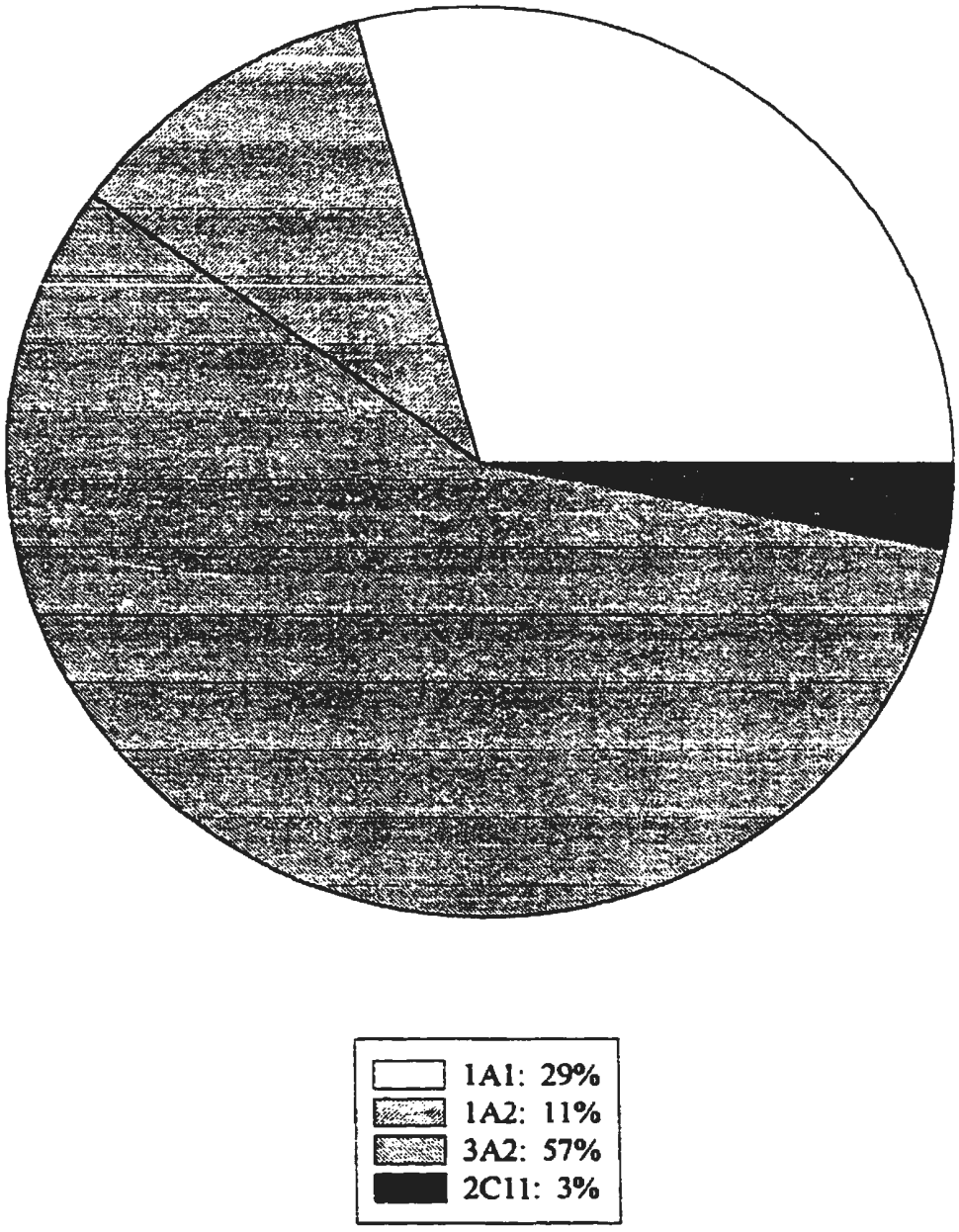


Figure 1.1.B. P-450 isozymes responsible for the 6β-hydroxylation of testosterone (Wood *et al.*, 1983; Ryan *et al.*, 1984a; Ryan *et al.*, 1984b).



1.2. Neonatal defeminization and adult masculinization of sex-specific P-450s

Sex-specific P-450s have been classified according to how they are regulated and/or 'programmed' in both the adult and neonatal rat. Many deactivating and/or bioactivating P-450 pathways are regulated by a number of environmental and/or hormonal factors. Einarsson *et al.* (1973) classify microsomal steroid metabolizing enzymes into three distinct groups: ***(a)*** *enzymes with a basal activity level regulated by nongonadal factors but reversibly inducible by androgens,* ***(b)*** *enzymes irreversibly "imprinted" or "programmed" by androgens during the prepubertal period and reversibly stimulated by androgens postpubertally,* and ***(c)*** *enzymes primarily regulated by nongonadal factors and only slightly affected by androgens.*

1.2.1. <u>Ontogeny of P-450 isozymes</u>: We examined two sex-specific P-450 isozymes typically categorized into group ***(b)***, specifically 2C11 and 3A2. These sex-specific P-450s are usually not dimorphically expressed until the beginning of puberty, and are not *fully* differentiated until the cessation of puberty (El Defrawy El Masry *et al.*, 1974; Waxman, 1984; Waxman *et al.*, 1985; Sonderfan *et al.*, 1987), puberty being defined as occurring between days 21 - 56 (Gram *et al.*, 1969). It is important to point out when the expression of these P-450s emerge (puberty), as this thesis deals with significant events based on *specific time-frames* in the life of the male rat.

EMDM (3A2): El Defrawy El Masry *et al.* (1974) showed that both the metabolism of ethylmorphine, and the apparent K_m (substrate affinity) of its associated demethylase (EMDM), lacked a significant sex difference in 21-day old rats. At the age of 28 days, males had an increased capacity to demethylate this compound, the maximum male capacity and optimal sex difference was seen at the age of 49 days. EMDM K_m was shown to decrease significantly from 21-35 days (Gram *et al.*, 1969), and was sexually differentiated by day 56 (male less than female) (El Defrawy El Masry *et al.*, 1974).

6β-hydroxylase (3A2): The 6β-hydroxylation of testosterone shows a slightly different developmental profile, reaching 72% of intact male activity by day 28 (Waxman *et al.*, 1988), perhaps due to possible differences in the developmental expression/regulation of the additional isozymes responsible for this reaction (**Figure 1.1.B.**). The sex differences in 6β-hydroxylation are probably not due to a developmental *induction* at puberty in the male, but instead, due to a developmental *suppression* in the female (Waxman *et al.*, 1985). This suppression occurs between days 28-60 (Sonderfan *et al.*, 1987), ultimately leading to male activity being 20-fold greater than that of females (Waxman *et al.*, 1985).

2α-hydroxylase and 16α-hydroxylase (2C11): The activities of 2α- and 16α-hydroxylase, also show an age-dependent increase in male rats, beginning at day 28 (Sonderfan *et al.*, 1987). At day 35, P-$450_{16\alpha}$ apoprotein[6] -expression in male rats reached 46% of the adult levels (Morgan *et al.*, 1985), while only 4% of the adult

[6] a protein composed in part of a polypeptide

activity of 2α-hydroxylase was reached by day 28 (Waxman *et al.*, 1988). Waxman (1984) demonstrated that adult activity of both the 2α- and 16α-hydroxylations of testosterone are reached by day 56.

Male rats have been shown to have 20-fold more hepatic steady-state 2C11 mRNA expression in adulthood, as compared with the female rat (Legraverend *et al.*, 1992b). The level of 2C11 mRNA was not detectable at day 14 (Maeda *et al.*, 1984), and began to increase significantly around day 21, reaching adult levels by 40-54 days (Maeda *et al.*, 1984; Morgan *et al.*, 1985; Janeczko *et al.*, 1990). Waxman *et al.* (1985) suggest a >30-fold induction of 2C11 protein levels at puberty in the liver of males, but not females. Thus, there is a definite peripubertal induction of 2C11 mRNA expression, consistent with the peripubertal induction (day 28-49) seen in 2α- and 16α- testosterone hydroxylase activity by Waxman (1984).

7α-hydroxylase (2A1): Levels of 2A1 and associated testosterone 7α-hydroxylase activity, have been demonstrated to be present at up to 4-fold higher levels in adult females as compared to adult males (Waxman *et al.*, 1985; Sonderfan *et al.*, 1987; Waxman *et al.*, 1989). The 7α-hydroxylation of testosterone in the male, was shown to be relatively high at four weeks of age (day 28), and then decreased by more than 75% by puberty (Sonderfan *et al.*, 1987). Levels of 2A1 mRNA have also been shown to be high in the immature rat, only to be suppressed (by up to 70%) by 56-70 days of age (Waxman *et al.*, 1985; Waxman *et al.*, 1988; Waxman *et al.*, 1989).

Aniline hydroxylase (2E1): The specific activity of aniline hydroxylase, is not sexually-differentiated in the rat (Kato *et al.*, 1968; MacLeod *et al.*, 1972; El Defrawy El Masry *et al.*, 1974; Finnen and Hassall, 1980). MacLeod *et al.* (1972) found that aniline hydroxylase activity increased from birth to five weeks (35 days) of age, when adult activities are reached, whereas Cresteil *et al.* (1986) indicate a peak in activity at day 15, followed by a slow decrease of 50%. Aniline hydroxylase V_{max} doubles between 1-2 weeks of age, followed by a significant decrease occurring between day 21-56 (Gram *et al.*, 1969). The developmental profile of P-450 2E1 is similar, male levels decreased by 60-75%, during weeks 2-8 (Waxman *et al.*, 1989).

Microsomal P-450 content and cytochrome c reductase: The liver itself undergoes significant growth spurts, especially between days 25-70 (Wilson and Frohman, 1974), essentially doubling in weight every week for the first five weeks, and then doubling again during puberty, between day 35-70 (MacLeod *et al.*, 1972). Microsomal protein has been shown to increase significantly between birth and day 21-25 (MacLeod *et al.*, 1972; Wilson and Frohman, 1974). MacLeod *et al.* (1972) indicated that adult levels of microsomal P-450 are reached by day 21 (males > females), Cresteil *et al.* (1986) suggesting that 50% of adult levels are reached by day one, and up to 70-80% of adult levels are reached by day 15. Hepatic cytochrome *c* reductase reached adult levels of activity around day 35, with no apparent sex difference (MacLeod *et al.*, 1972). However, Wilson and Frohman (1974) demonstrated that cytochrome *c* reductase activity increases from birth to day 70.

1.2.2. Neonatal defeminization: The peripubertal emergence (or "activation") of sex-specific P-450s is "organized" or "imprinted" during a defined neonatal period (Denef and DeMoor, 1968a,b; Einarsson *et al.*, 1973; Gustafsson and Stenberg, 1974a,b; Chung, 1977; Gustafsson *et al.*, 1983). This permanent process is often referred to as an *organizational* event, and appears to involve two distinct components. Neonatal imprinting of sex-specific P-450s is an event which ***permanently increases the activity/amount of P-450 as a result of specific neonatal sex-steroids*** and also results in a ***permanent ability of a P-450 to respond post-pubertally*** (*i.e.* by an increase in specific activity) ***to testosterone***. It is also referred to as *defeminization*, as it results in the suppression of both behavioural and neuroendocrine functions characteristic of the *female*, and consequently results in the expression (or programs the ability for post-pubertal expression) of *male*-specific P-450s. We will subsequently refer to this neonatal imprinting process, as **neonatal defeminization**.

Prevention of neonatal defeminization: Prevention of neonatal defeminization (*i.e.* by neonatal castration) has a profound effect on the post-pubertal emergence of sexually dimorphic P-450s. For example, in the absence of neonatal defeminization, both the K_m and V_{max} of EMDM increased and decreased respectively, towards a female value (Chung, 1977; Reyes and Virgo, 1988). The protein levels of 2C11 and 3A2, as well as their respective metabolic reactions (ethylmorphine demethylation and the 16α-, 2α-, 6β-hydroxylations of testosterone), also do not attain post-pubertal adult male levels, when neonatal defeminization has

been prevented by castration (Finnen and Hassall, 1980; Kamataki *et al.*, 1983; Dannan *et al.*, 1986; Shimada *et al.*, 1987; Waxman *et. al.*, 1989; Cadario *et al.*, 1992; Bandiera and Dworschak, 1992).

Exogenous neonatal defeminization: Neonatal defeminization can be effected however, if testosterone, estradiol, or dihydrotestosterone (DHT), are administered during a 'critical' time frame (Gustafsson and Stenberg, 1976; Gustafsson *et al.*, 1977; Reyes and Virgo, 1988). For example, the critical period for neonatal defeminization of EMDM K_m is suggested to be days 2-4, whereas the V_{max} of this enzyme may not be completely defeminized until day 12-14. Testosterone therapy administered to neonatal castrates *during these specific time frames* can effectively 'rescue' these sexually differentiated parameters of EM metabolism (Chung, 1977). Neonatal testosterone therapy to the non-defeminized male (neonatally castrated), partially restored 3A2 (EMDM or 6β-hydroxylase) activity, and/or 2C11 activities (Kamataki *et al.*, 1986; Dannan *et al.*, 1986; Shimada *et al.*, 1987; Waxman *et al.*, 1989; Virgo, 1991). Castration at any time *after* day 5, resulted in *higher* basal[7] activities of EMDM than castration *before* day 5 (Finnen and Hassall, 1980), indicating that the defeminization of basal enzyme activity occurred before day 5.

Neonatal castration did not affect the ultimate emergence of aniline hydroxylase activity (Chung *et al.*, 1975; Finnen and Hassall, 1980; Shimada *et al.*, 1987), and may either slightly increase (Dannan *et al.*, 1986; Shimada *et al.*, 1987;

[7] not stimulated by testicular secretions

Waxman *et al.*, 1989) or have no effect (Waxman *et al.*, 1985; Cadario *et al.*, 1992) on the adult activity of 7α-hydroxylase. This suggests that these reactions are probably not neonatally defeminized by testicular secretions.

P-450 responsiveness to testosterone: Neonatal testosterone therapy (exogenous defeminization), also results in an ability of EMDM activity to be stimulated (*masculinized*) by testosterone in adulthood, a characteristic absent in a non-defeminized male (Chung *et al.*, 1975; Virgo, 1991).

1.2.3. Adult masculinization: Whereas *organizational* events are permanent events which occur neonatally, *activational* events are those which are *not* permanent, occur *post-pubertally*, and are *reversible* in the absence of specific hormones. This non-permanent response is also termed *masculinization*, as this results in the ***enhancement of both the previously organized behavioural and neuroendocrine patterns characteristic of the male***, and may ultimately lead to the ***appearance of, or increase in, the activity of previously organized (defeminized) P-450 expression***. For example, adult castration significantly decreases 2C11 mRNA, but this effect is completely reversible upon methyltrienolone (androgen) therapy (Janeczko *et al.*, 1990).

Castration in adulthood also has been shown to decrease EMDM activity by 35-80% (El Defrawy El Masry and Mannering, 1974; Kramer *et al.*, 1975b; Virgo, 1991), the effect being either completely reversed by androgen therapy (El Defrawy El Masry and Mannering, 1974; Kramer *et al.*, 1975b), or stimulated by somatostatin

therapy (Virgo, 1985). Adult castration has also been shown to increase EMDM K_m, which was reversible by testosterone therapy (El Defrawy El Masry and Mannering, 1974). Dihydrotestosterone (DHT) treatment (Kramer *et al.*, 1979) or methyltrienolone treatment (Janeczko *et al.*, 1990) of the adult castrate *also* partially restored EMDM activity and 2C11 levels respectively, suggesting that the masculinization process may be androgen-mediated. This is most likely true, as estradiol administration to the adult castrate has been shown to *decrease* EMDM activity (Kramer *et al.*, 1979).

Sex hormones have also been shown to modify P-450 activity in the intact male. Estradiol administration to the intact male has been shown to increase EMDM K_m (Kramer *et al.*, 1979), eliminate expression of 2C11 mRNA (Janeczko *et al.*, 1990), and decrease 2C11 content (Kamataki *et al.*, 1986). Diversion of portal blood away from the liver (which increased circulating estradiol concentrations and decreased testosterone concentrations), significantly decreased 2C11 and 3A2 mRNA expression (Jiang *et al.*, 1994).

1.3. The physiology of neonatal defeminization

Neonatally castrated adult male rats, exhibit patterns of steroid and xenobiotic metabolism characteristic of the female (thus, they are not defeminized). The elimination of the source of testosterone (the testes) within 24-hours of birth ultimately prevents the peripubertal dimorphism of P-450s. Although neonatal

castration abolishes sex-specific expression of certain isozymes, the administration of specific sex steroids (testosterone, estrogens, DHT) during the 'critical period' (day 0-14) can effect neonatal defeminization of these isozymes (Gustafsson and Stenberg, 1976; Gustafsson *et al.*, 1977; Reyes and Virgo, 1988). Studies by Reyes and Virgo (1988) supply evidence that neonatal defeminization of P-450 3A2 most likely occurs through the estrogen receptor, as blocking this receptor with Nafoxidine™ resulted in the K_m and V_{max} of EMDM decreasing and increasing respectively to values not different from intact females.

1.3.1. Aromatase: Aromatase is the enzyme complex that catalyzes the conversion of androgens into estrogens. It is bound to the endoplasmic reticulum of the cell, and is comprised of two proteins, cytochrome $P\text{-}450_{arom}$ and NADPH-cytochrome P-450 reductase (Brueggemeier, 1994). Aromatase activity is very high in the male rat hypothalamus at day 20 of gestation, dropping to low levels by postnatal day 16-20 (George and Ojeda, 1982). Other areas of the neonatal rat brain exhibiting aromatase activity include areas of the *(a)* preoptic nucleus, *(b)* amygdaloid nucleus, and *(c)* bed nucleus of the stria terminalis (Shinoda *et al.*, 1994). This corresponds with the appearance of estrogen formation in the hypothalamus (also including the preoptic area, septum, and amygdala), which increases during embryonic days 15-19 and declines thereafter (MacLusky *et al.*, 1985). The same study indicated that males demonstrated higher amounts of estrogen formation than females, on postnatal days 1-4 (MacLusky *et al.*, 1985), and this also correlated with

the developmental expression of the hypothalamic estrogen receptor (Pasterkamp *et al.*, 1996). Hypothalamic estradiol concentrations have been shown to increase dramatically between 0 hour *in utero*, and 1 hour after delivery, decreasing thereafter (Rhoda *et al.*, 1984).

1.3.2. <u>1,4,6-androstatriene-3,17-dione (ATD)</u>: The aromatase enzyme is particularly important to the work presented in this thesis, as this is the enzyme complex which we will *inhibit* in order to prevent the neonatal defeminization of sex-specific P-450s. Reyes and Virgo (1988) demonstrated the ability of an aromatase inhibiting agent 1,4,6-androstatriene-3,17-dione (ATD) to prevent the conversion of testosterone to estradiol, and thus prevent the neonatal defeminization of EMDM (P-450 3A2). They successfully demonstrated that blocking the aromatase enzyme, instead of blocking the estrogen receptor, could also prevent the testosterone propionate-induced neonatal programming of EMDM, but could not prevent the defeminization brought about by estradiol (which does not undergo aromatization). It was speculated that testosterone does not defeminize through the *androgen* receptor, due to the observation that this hormone must first be converted to estradiol in order to effect defeminization.

Due to the nature of the experiments described in this thesis, ATD (as an alternative to neonatal castration), was employed as an aromatase blocker to pharmacologically prevent neonatal defeminization of sex-specific P-450s 3A2 and 2C11. ATD is a mechanism-based inhibitor of aromatase, meaning that it *(a)* mimics

the substrate, *(b)* is converted by the enzyme to a reactive intermediate, and *(c)* results in the permanent inactivation of the enzyme (Brueggemeier, 1994). ATD has been shown to block the testosterone-induced increase in aromatase mRNA concentration (in the castrated quail), and is suggested to block the synthesis of aromatase at the transcriptional level (Foidart *et al.*, 1995).

It is extremely important to keep the hypothalamo-pituitary-gonadal axis intact, in order to maintain the endocrine status as 'normal' as possible. Most research on P-450 defeminization/masculinization has been performed on neonatally castrated males, so our approach was unique.

1.3.3. Defeminization of the male rat brain: Research has been successful in only correlating the ultimate expression of sex-specific P-450s to the neonatally programmed, sexually dimorphic, rat brain. Central nervous system (CNS) characteristics subject to organizational effects of perinatal gonadal hormone exposure include gonadotrophin release patterns (Vreeburg *et al.*, 1977), behaviour (both non-reproductive and reproductive) (Vreeburg *et al.*, 1977; Korenbrot *et al.*, 1975), and brain morphology (Dorner and Staudt, 1968; Gorski *et al.*, 1978; Arnold and Gorski, 1984; Handa *et al.*, 1985; Davis *et al.*, 1996). Estradiol and testosterone have both been shown to be effective at imprinting the brain as *masculine* (with respect to regulation of gonadotrophin release and estrogen/progesterone-primed lordosis), whereas DHT (a non-aromatizable substrate) does not imprint these characteristics (Korenbrot *et al.*, 1975).

Sex hormone receptors: Neonatal castration of the male, has been shown to significantly feminize many areas of the rat brain. For example, the number of nuclear testosterone-binding sites in the pituitary, mediobasal hypothalamus, and the preoptic anterior hypothalamus, has been shown to decrease following neonatal castration (Babichev *et al.*, 1990). In addition, the same study indicated that the number of nuclear estrogen receptors decreased following neonatal castration in the mediobasal hypothalamus, and increased in the preoptic anterior hypothalamus. DonCarlos *et al.* (1995) also demonstrated that neonatal castration increases estrogen receptor mRNA, to female levels, in the preoptic area, suggesting that testosterone-derived estrogen down-regulates estrogen receptor mRNA in the neonatal male hypothalamus (preoptic area).

Brain morphology: The volume of a number of areas of the male rat brain, particularly in the hypothalamus, have also been shown to be affected (feminized) by neonatal castration. Neonatal castration significantly decreased (feminized) the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Gorski *et al.*, 1978; Handa *et al.*, 1985; Davis *et al.*, 1995), and increased (feminized) both the volume of the hypothalamic ventromedial nucleus (Dorner and Staudt, 1969) and anteroventral periventricular nucleus (Davis *et al.*, 1996), in adulthood. Neonatal castration also significantly increased the estrogen-primed colocalization of galanin and luteinizing-hormone-releasing-hormone (LHRH), in the LHRH-containing neurons of the male hypothalamus, to levels similar to the female (Merchenthaler *et al.*, 1993).

Behaviour: Masculine behaviour is also affected by neonatal castration, as this treatment increased estrogen (and progesterone) induced lordosis, feminine proceptive behaviour, and decreased mounting and/or intromission frequency (Bloch and Mills, 1995). In response to estrogen and/or progesterone priming in adulthood, neonatal castration effected an increase in the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Handa *et al.*, 1985). Infusion of an antisense oligodeoxynucleotide to estrogen receptor mRNA (on day 3 of life) to the female, prevented testosterone-induced masculinization of sexual behaviour, and also prevented complete feminization of the volume of the SDN-POA (McCarthy *et al.*, 1993), indicating that the estrogen receptor is involved in sexual differentiation of these phenomena.

1.3.4. Mechanism of defeminization: Support for the "aromatization-defeminization" concept is presented in literature regarding the use of ATD to prevent *behavioural* defeminization of the male rat. Perinatally administered ATD has been shown to block testosterone propionate-, but not dihydrotestosterone (DHT)- or estradiol benzoate-induced copulatory behaviour in sexually inexperienced castrated adult male rats (Morali *et al.*, 1977), demonstrating the requirement of the aromatase enzyme in the induction of masculine sexual behaviour by androgens. Partner preference behaviour (sexual interaction with an estrous female) and sexual behaviour (*i.e.* hopping, darting, mounting, intromission) in both gonadally intact and testosterone-primed castrated male rats has been altered to a nocturnally rhythmic

'bisexual' status by the perinatal administration of ATD (Bakker *et al.*, 1995). Finally, perinatally administered ATD has been shown to inhibit sulphated cholecystokinin octapeptide (sCCK-8) induced lordosis in neonatally castrated males (Ulibarri and Micevych, 1993), and to effect 'bisexual' partner preference behaviour in male rats (Swaab *et al.*, 1995).

It is of interest that perinatally administered ATD has been shown to cause an increase in vasopressin-expressing neurons in the suprachiasmatic nucleus of male rats (Swaab *et al.*, 1995) suggesting that the volume dimorphism seen in the Gorski *et al.* (1978) study may be programmed perinatally by the aromatization of androgens to estrogens.

The observed central nervous system sex differences are relevant to our studies for three reasons: **(a)** a number of these differences have been shown to be neonatally defeminized by testicular steroids; **(b)** neonatally defeminized areas of the rat brain may be associated with the expression of the sexually dimorphic growth hormone (GH) secretion pattern in the adult rat (for review see Jansson *et al.*, 1985b); and **(c)** this dimorphic GH pattern may be responsible for the adult expression of certain neonatally defeminized sex-specific P-450s.

1.4. Sexually dimorphic growth hormone secretion patterns

The most significant brain function known with regard to sex-specific hepatic P-450s, is the maintenance of a sexually dimorphic growth hormone (GH) pattern, as

it is this dimorphism which has been suggested to ultimately regulate the sex-specific expression of 3A2 and 2C11.

Ontogeny: The GH secretory pattern has been shown to be episodic in both males and females at the age of 22 days, the peak levels never reaching adult levels in either sex (Eden, 1979). Serum GH concentrations are high at birth and shortly thereafter, decreasing to low levels by day 25. GH concentrations subsequently increase significantly (8-fold) from day 25-45 in the rat, and again (5-fold) by day 90 (Gabriel *et al.*, 1989), the sexual dimorphism in secretory pattern becoming apparent between day 30-90 (Eden, 1979). This corresponds with the appearance of sex-specific P-450s (El Defrawy El Masry *et al.*, 1974; Waxman, 1984; Morgan *et al.*, 1985; Waxman *et al.*, 1985; Sonderfan *et al.*, 1987).

Male growth hormone pattern: Males display high (200-300 ng/ml) amplitude pulses at regular 3-4 hour intervals, separated by low trough values (<5 ng/ml) (Eden, 1979).

Female growth hormone pattern: When adulthood is reached, female rats exhibit GH pulses that are irregular, more frequent, and of lower height than those of the male. Most importantly, the GH secretion pattern is of a continuous nature, with significantly higher baseline levels than the male (Eden, 1979).

1.4.1. GHRH and SS signaling to the pituitary: Literature indicates that there are sex-differences in the mode of somatostatin (SS)-releasing factor and growth hormone releasing hormone (GHRH) signaling to the pituitary (Painson and

Tannenbaum, 1991) and GH feedback in the median eminence (ME) and hypothalamus (Maiter *et al.*, 1990). Maiter *et al.* (1990) showed that male rats have a *higher* degree of sensitivity in GH feedback to the ME and hypothalamus than female rats, as indicated by a decrease in GH secretion and an increase in SS secretion in the presence of GH. Basal GHRH gene expression in the hypothalamus is higher in males, and its expression in the male hypothalamus is more sensitive to feedback inhibition by growth hormone (Maiter *et al.*, 1991). It has also been suggested that there is a time-dependent difference in the response of GH somatotrophs to GHRH (Painson and Tannenbaum, 1991). Carlsson *et al.* (1990) demonstrated a 3-hour pattern of intermittent *response* of GH to GHRH in the male.

Regardless of how the differences in secretory patterns are effected at a physiological level, it is important to note that: **(a)** the dimorphism is not seen until the onset of puberty, and **(b)** like the sex-specific P-450s, certain characteristics of the male secretory profile appear to be neonatally defeminized, whereas other characteristics appear to be directly regulated in adulthood.

1.4.2. Neonatal defeminization of dimorphic GH secretion patterns:

Neonatal castration (prevention of defeminization), effects a feminine pattern of growth hormone secretion in adulthood, *i.e.* a 50-75% reduction in GH pulse height and duration (Jansson and Frohman, 1987), and a significant increase in baseline levels (Mode *et al.*, 1982; Jansson *et al.*, 1985a; Jansson and Frohman,

1987). Neonatal gonadectomy also suppressed maximum and mean plasma GH levels during adult life (Jansson *et al.*, 1985a).

Prepubertal gonadectomy of the defeminized male, on the other hand, did *not* suppress maximum and/or mean plasma GH levels in adulthood (Jansson *et al.*, 1985a; Carlsson *et al.*, 1987), but significantly increased baseline (minimum) GH levels (Gustafsson *et al.*, 1983; Carlsson *et al.*, 1987). Neonatal androgen replacement (exogenous defeminization) only *partially* masculinizes the adult GH secretion pattern. It has been suggested that neonatal testosterone is sufficient to restore the high amplitude GH pulses (Jansson and Frohman, 1987). The pulses, however, were of significantly shorter duration and occurred more frequently, than those seen in the adult male rat. More importantly, the baseline GH levels remained significantly higher than that of the intact males (Jansson *et al.*, 1985a; Jansson and Frohman, 1987).

Thus, neonatal testosterone therapy is sufficient to organize the normal post-pubertal pulse *amplitudes*, but is not sufficient to restore low baseline levels.

Both neonatal and adult testosterone therapy of the neonatal castrate, on the other hand, resulted in GH patterns indistinguishable from the intact male (Jansson and Frohman, 1987). This suggests that adult androgens are necessary for the low baseline levels, as well as the pulsatile nature of the male GH secretory pattern. Estradiol administration to the adult male, intact or castrated, also significantly increased baseline GH levels (Gustafsson *et al.*, 1983; Carlsson *et al.*, 1987), in a dose-dependent manner (Gustafsson *et al.*, 1983). Estradiol treatment of the intact

male, has also been shown to lower the magnitude of the GH peaks, and cause them to occur less regularly (Gustafsson *et al.*, 1983).

It is of interest that adult testosterone therapy (to the non-defeminized neonatal castrate) was sufficient to effect a fully masculine GH secretory pattern (Jansson and Frohman, 1987). This suggests that neonatal androgens are *effective* but are not *necessary*, to defeminize/masculinize characteristics of the GH secretory profile.

1.4.3. <u>Adult regulation of dimorphic GH patterns</u>: Previous literature demonstrates that sex hormones do not *directly* regulate the expression of sex-specific P-450s, but require an intact pituitary gland to exert their effects (Kramer *et al.*, 1975b; Kramer *et al.*, 1979; Kamataki *et al.*, 1985). This most likely occurs through the modulation of the growth hormone secretory profile, as manipulation of sex hormones has been shown to affect the pattern of growth hormone secretion (Mode *et al.*, 1982; Gustafsson *et al.*, 1983; Jansson *et al.*, 1985a; Carlsson *et al.*, 1987). For example, castration of the adult male resulted in a significant decrease in EMDM specific activity (Kramer *et al.*, 1975b), and this decrease could be reversed by testosterone therapy (Kramer *et al.*, 1975b), or partially reversed by DHT administration (Kramer *et al.*, 1979). Estradiol administration to the non-defeminized male, has been shown to significantly decrease EMDM specific activity (Kramer *et al.*, 1978; Kramer *et al.*, 1979), in a dose-dependent manner (Kramer *et al.*, 1978). However, Kramer *et al.* (1979) found that, in the absence of the pituitary

gland, estradiol or DHT administration to the male castrated in adulthood, had *no* effect on EMDM activity.

1.4.4. Exogenous GH and restoration of P-450 activity:

EMDM: In castrated males, GH administration (twice daily) has been shown to significantly decrease EMDM activity (Kramer *et al.*, 1978; Virgo, 1985), an effect also demonstrated in the intact male (Kramer *et al.*, 1975a; Kramer *et. al.*, 1978). Estradiol administration to the castrated male *further* decreased EMDM activity (Kramer and Colby, 1976; Kramer *et al.*, 1978), and growth hormone administration had the same effect (Kramer and Colby, 1976), supporting the concept that estradiol is affecting EMDM activity through modulation of the growth hormone secretory pattern. Somatostatin therapy to the castrated male rat, has also been shown to stimulate EMDM activity (to almost the same degree as testosterone administration), and the concomitant administration of both hormones revealed a synergistic interaction (Virgo, 1985).

6β-hydroxylase: Hypophysectomy of the adult male has been shown to effect a significant *increase* in 6β-hydroxylase activity (Yamazoe *et al.*, 1986b; Kato *et al.*, 1986), as well as 6β-hydroxylase mRNA (Shimada *et al.*, 1989). When given to hypophysectomized males, either intermittent or continuous GH therapy significantly decreased the induction in activity/mRNA (Yamazoe *et al.*, 1986b; Shimada *et al.*, 1989), whereas Kato *et al.* (1986) found that GH administration (twice daily) did *not* decrease the induction brought about by hypophysectomy,

however continuous infusion of GH was effective at reversing the induction. Continuous infusion of GH to the pituitary-intact male significantly decreased 6β-hydroxylase activity (Waxman *et al.*, 1989; Kato *et al.*, 1986). Shimada *et al.* (1989) reported that growth hormone most likely regulates P-450 6β-hydroxylase at a pretranslational step.

3A2 mRNA levels: Hypophysectomy also effected a significant increase in hepatic immunoreactive P-450 3A2 (Waxman *et al.*, 1988; Waxman *et al.*, 1990). Intermittent injection of human growth hormone (hGH) to hypophysectomized males partially reversed the induction of 3A2 (Waxman *et al.*, 1988). Continuous infusion of hGH to the hypophysectomized male *almost* completely abolished 3A2 mRNA levels (Waxman *et al.*, 1990), whereas continuous GH *completely* abolished 3A2 mRNA in *intact* males (Waxman *et al.*, 1990). This also suggests that other pituitary factors may be required for complete suppression of P-450 3A2.

2α-/16α-hydroxylases: Hypophysectomy significantly decreased 2α- and 16α-hydroxylation of testosterone, whereas intermittent injection (twice daily) of GH restored intact male levels of these reactions (Yamazoe *et al.*, 1986a,b). Morgan *et al.* (1985) also found that hypophysectomy of male rats causes a decrease in 16α apoprotein levels to values intermediate to those of the intact males and females, and continuous infusion of GH decreased 16α apoprotein levels to female levels. Continuous GH administration, to either the intact or hypophysectomized male, caused a significant decrease in 16α mRNA levels (Strom *et al.*, 1987).

Lesions of the anterior hypothalamic periventricular area (which decreased median eminence somatostatin content), also decreased 16α-hydroxylase activity (Norstedt *et al.*, 1983).

2C11 expression diminished greatly after hypophysectomy of male rats and was markedly stimulated by intermittent GH therapy (Waxman *et al.*, 1989; Janeczko *et al.*, 1990; Waxman *et al.*, 1991; Legraverend *et al.*, 1992a). Waxman *et al.* (1991) found that subcutaneous GH pulses delivered six times daily, as well as by the less frequent, non-physiological frequencies of two and four times daily, were equally effective in restoring 2C11 expression. A dramatic difference (increase) was discovered in the responsiveness of 2C11 to six *vs.* seven GH pulses per day, which strongly suggests that to effect a male liver response, the minimal plasma GH trough time needed is at least 2.5 hours. Legraverend *et al.* (1992b) suggest that neither the amplitude nor the frequency of the GH pulse is recognized as male or female by the hepatocyte, but the hepatocytes are responding to the prolonged suppression of circulating GH during the trough period.

Sundseth *et al.* (1992) used both untreated and hypophysectomized adult male rats to demonstrate that GH (either continuously infused or intermittently injected) regulates the sex-specific expression of the 2C11 genes at the level of transcription initiation. Intermittent injection increased 2C11 mRNA expression, infusion decreased it. Mode *et al.* (1989b) found that P-450 16α mRNA decreases in hypophysectomized adult males, and is restored by intermittent hGH administration (twice daily), and all of these changes occur at the pretranslational level. Continuous

hGH exposure, on the other hand, caused a significant reduction in 16α mRNA levels to those found in the female. To clarify even further, Legraverend *et al.* (1992a) found that 2C11 mRNA expression reaches the same level as that in intact males 24 hours after the second daily injection of hGH and induces transcription of the 2C11 gene at a level comparable to that in normal males within three days. They confirm that sex specificity is tightly controlled at the transcriptional level and that two clearly distinct patterns of transcriptional regulation by GH emerge.

Growth hormone binding/receptors: Wells *et al.* (1994) found that pulsatile (male) GH therapy lowered the percent specific binding of GH to hepatic (lactogenic and somatogenic) GH receptors, and continuous GH infusion increased binding. Therefore, it is likely that the GH receptor is occupied *more* continuously in the case of the female, and *less* continuously in the case of the male. Perhaps the increased length of time that the GH molecule is bound to the receptor, serves to signal for an increase in female-specific P-450 expression, and vice-versa for the male-specific P-450s. Growth hormone can form 1:1 or 1:2 complexes with GH receptors, and the 1:2 complexes can be dissociated by higher concentrations of GH (Cunningham *et al.*, 1991). Wells *et al.* (1994) suggest that these different complexes could mediate different signals over different ranges of GH concentrations. Baumbauch and Bingham (1995), characterize one class of hepatic growth hormone receptor (GHR_1) as being sexually dimorphic, and regulated by GH. Castration of the male resulted in an induction of steady-state GHR_1 RNA. Continuous infusion of growth hormone actually *further* increased the GHR_1 levels, to levels similar to the intact female.

Cytochrome P-450 content: Wilson (1973) demonstrated that GH administration to the intact male, decreased total microsomal P-450 content by 46%, and Wilson and Frohman (1974) showed that high levels of GH were correlated with low levels of P-450 components (P-450 content and cytochrome *c* reductase activity). On the other hand, Virgo (1985) did not demonstrate any effect of GH administration (twice daily) on either microsomal protein or total P-450 content.

Cytochrome c reductase: A significant decrease was noted in cytochrome *c* reductase activity, following GH administration (twice daily) (Kramer *et al.*, 1978; Virgo, 1985), suggesting that reductase is partially regulated by GH. Kramer *et al.* (1978) also found that the administration of estradiol at higher doses caused a significant decrease in reductase activity, and Virgo (1985) found that testosterone and/or somatostatin administration had the reverse effect. To further confirm that the effects of testosterone were mediated through the pituitary gland (and possible modulation of GH secretion), Virgo (1991) found that testosterone could not increase cytochrome *c* reductase activity in the absence of the pituitary gland, and GH infusion reversed the stimulation of reductase by testosterone.

1.4.5. Summary: Continuous infusion of GH to the intact or hypophysectomized male, caused a significant suppression of 6β-hydroxylase (and associated 3A2 levels) and 16α-hydroxylase activities (Yamazoe *et al.*, 1986a,b; Morgan *et al.*, 1985; Waxman *et al.*, 1990). The absence of GH altogether resulted in a significant increase in 6β-hydroxylase activity (Yamazoe *et al.*, 1986b), whereas it caused a

decrease in EMDM and 16α-/2α-hydroxylases (Kramer *et al.*, 1975a,b; Yamazoe *et al.*, 1986a). GH administration in the male pattern, has been shown to restore 2α-/16α-hydroxylase activities and 2C11 levels (Yamazoe *et al.*, 1986a), provided the trough period is at least 2.5 hours in length (Waxman *et al.*, 1991). The male GH pattern decreased both EMDM and 6β-hydroxylase activity (Yamazoe *et al.*, 1986b; Kramer *et al.*, 1978). Thus, it appears that the male expression of 2C11 (and associated 2α-/16α-hydroxylations) is *absolutely dependent* on the male growth hormone pattern, being regulated at pretranslational levels (Mode *et al.*, 1989b). The adult expression of EMDM and 6β-hydroxylase activities, on the other hand, are suppressed by continuously high levels of GH, but the absence of GH suppressed EMDM and induced 6β-hydroxylase activity (Kramer *et al.*, 1975a,b; Yamazoe *et al.*, 1986b). The male pattern of GH, has not been conclusively determined to regulate either EMDM or 6β-hydroxylase, but it appears that their regulations are under different control than 2C11.

1.5. On the pubertal time-frame

The critical time frame for the imprinting of sex-specific cytochrome P-450 3A2 (EMDM) by hormonal manipulations, is from birth to approximately day 14 (Chung, 1977). If defeminization does not occur during this time, then exposure of the non-defeminized male to androgens or estrogens has been shown to have *no effect* on the activity of this isozyme (Chung *et al.*, 1975; Virgo, 1991). Suprisingly, recent

studies have demonstrated that in neonatally castrated males (*i.e.* non-defeminized), exposure to androgens (*i.e.* testosterone) during the peripubertal period (day 35-70) can completely masculinize both 2C11 and 3A2 (Shimada *et al.*, 1987; Virgo, 1991; Waxman *et al.*, 1989; Dannan *et al.*, 1986). This phenomenon has also been demonstrated in the ovariectomized female rat (Cadario *et al.*, 1992)

1.5.1. Peripubertal masculinization of the male: Dannan *et al.* (1986), present data on the effects of neonatal castration and subsequent testosterone treatment (day 35-70), on P-450 2C11 and 3A2 protein levels, and also on 16α-hydroxylase and 6β-hydroxylase specific activities, in adulthood. They found that testosterone administration (Silastic™ capsules) restored intact male levels of both 2C11 and 3A2 protein, as well as their respective hydroxylations. Testosterone administration on day 1 and 3 of life, only restored defeminized levels of these proteins and activities.

During studies on the persistence of neonatal androgen defeminization of P-450s 2C11 (2α- and 16α-hydroxylases) and 3A2 (EMDM), Shimada *et al.* (1987) discovered that testosterone treatment, from day 56-63, in the neonatal castrate, *completely* restored 2α-hydroxylase activity to that of intact males, and almost restored the 16α-hydroxylase and EMDM activities. It seems that 6β-hydroxylase activity was not as responsive during puberty, as testosterone treatment during this time-frame resulted in a partial increase, to only ~75% of intact (defeminized) male levels.

Waxman *et al.* (1989) presented similar data. They found that in neonatally gonadectomized males, testosterone-packed Silastic™ capsules implanted from day 35-70, resulted in a 5-fold increase in 16α-hydroxylase specific activity in adulthood (as compared with the birth castrate control).

Virgo (1991) subsequently studied the effects of peripubertal (day 35-71) testosterone treatment in neonatally castrated males, on the specific activity of P-450 3A2 (EMDM) in adulthood. He found that testosterone propionate treatment of the neonatal castrate, from day 35-71, increased EMDM activity to intact male levels, and this effect was completely reversed upon removal of the androgen. These effects required the presence of an intact pituitary gland, as hypophysectomy abolished them.

In 1992, Bandiera and Dworschak studied the effects of testosterone on the hepatic levels of P-450 2C11, in the male rat. They found that testosterone propionate given to the neonatal castrate, from day 56-70 (postpubertal), increased 2C11 content to intact male levels. They did not find any *increase* in males administered TP from days 35-49, however, assuming the animals were killed on day 70 (as were the shams and the neonatal castrate), these males were free of serum testosterone at the time of death. Therefore, these results represent *uninduced* (*i.e.* female, non-defeminized) 2C11 levels. Virgo (1991) suggested that this pubertal phenomenon was a reversible process, thus it would have been of interest to determine the level of 2C11 in the day 35-49 treated males, killed on day 50, instead of day 70.

1.5.2. <u>Peripubertal masculinization of the female</u>: There have been a number of studies performed on the ovariectomized female rat with regard to peripubertal "imprinting" 2C11 and 3A2 activity. Whether or not we can relate results from the female to that of the male is debatable, however evidence presented may add small pieces to the overall picture.

In 1984, Pak *et al.* found that exposure of female rats to testosterone enanthate during the pubertal period (day 35-50), resulted in increased sensitivity to androgens (testosterone enanthate) in adulthood. Ovariectomized (28 days of age) females responded even more in adulthood. These results indicate that androgens are the necessary "imprinting" factor, and estrogens were found to *antagonize* this effect. They did not find any imprinting of *basal* levels of aryl hydrocarbon hydroxylase (AHH), another male-predominant reaction (Wiebel and Gelboin, 1975), but the adult *responsiveness* to androgens *was* imprinted. In 1985, the same laboratory of Pak *et al.* also demonstrated that the female-specific testosterone 5-reductase (not a P-450) showed similar results in non- and ovariectomized (day 28) female rats exposed to testosterone from day 35-50. It appeared that pubertal testosterone was important for the expression of testosterone responsiveness in adulthood (again, the response was potentiated in ovariectomized animals).

Peripubertal testosterone treatment in the female has also been shown to induce *male*-specific P-450s. Testosterone given in both pubertal (day 35-42) and adult periods (day 63-70) induced male-specific acetohexamide reductase activity in liver microsomes of female rats, whereas treatment of the adult alone, was without

effect (Imamura *et al.*, 1994). In addition, gonadectomy at age 25 days, followed by testosterone enanthate administration from days 35-59, effected a complete appearance and masculinization (as opposed to the non-androgenized female) of both 2α- and 16α-hydroxylase in females (Cadario *et al.*, 1992).

The above evidence suggests that neonatal defeminization is **not** a necessary prerequisite for masculinization of sex-specific P-450s. It also suggests that there may exist a unique peripubertal time-frame in which certain sex-specific P-450s can be imprinted to specific activities not different from that of the intact adult male.

1.6. Summary

The isozymes responsible for the 16α(2C11)-, 2α(2C11)-, and 6β(3A2)-hydroxylation of testosterone, as well as the metabolism of ethylmorphine (3A2) are specifically expressed in the male rat. The isozymes are categorized as enzymes *irreversibly defeminized by androgens during the prepubertal period and reversibly stimulated by androgens postpubertally* (Einarsson *et al.*, 1973). Defeminization occurs during a critical time period (day 0-14) and results in a permanent increase in the activity/amount of P-450 present, and also may result in a permanent ability of a P-450 to respond postpubertally to testosterone (indicated by an increase in specific activity). After the critical time period has passed, a non-defeminized male (*i.e.* castrated) will exhibit a decreased postpubertal expression of 2C11 and 3A2, and will not respond (*i.e.* increase in specific activity) to testosterone (masculinization) in

adulthood. Postpubertal castration of a neonatally defeminized male will result in a partial loss of sexually dimorphic P-450 expression.

Neonatal defeminization is most likely effected by androgen-derived estrogens which act through the estrogen receptor. The aromatase enzyme is responsible for the conversion of androgens to estrogens.

Male-specific expression of some P-450s is regulated by a sexually dimorphic pattern of growth hormone secretion, this pattern is both neonatally defeminized by androgens/estrogens and regulated by sex-hormones in adulthood. Thus, the sex-specific expression of P-450s may represent an "end-point" or "marker" characterizing the sexual differentiation of this characteristic of the rat brain.

1.7. Hypothesis

Although neonatal defeminization has widely been considered a *necessary prerequisite* for adult masculinization of 2C11 and 3A2, data from a number of laboratories indicate that **peripubertal administration of testosterone to the neonatally castrated male, effects full masculine activities of these enzymes in adulthood**.

We suggest that the presence of intact testes from days 35 to 70 can effectively increase the amounts and/or activities of P-450 2C11 and 3A2 to those found in intact males. We also suggest that this is either an acute "masculinization" process (*i.e.* reversible after the removal of the testes) or a permanent defeminization

followed by masculinization (*i.e.* similar to neonatal defeminization). In other words, a basal level of activity is permanently imprinted, and this level can be further increased by testicular secretions in the adult animal.

For our experiments, we desired that the neonatal defeminization of male-specific cytochrome P-450 isozymes, in particular 2C11 and 3A2, be blocked by a mechanism other than neonatal castration. Therefore, we would not be disrupting the *entire* hypophyseal-pituitary-gonadal axis, and would be creating conditions more similar to those found in an intact animal.

We investigated the effect of intact testes during specific time frames in the peripubertal window, in particular days **21-35**, **21-55** and **21-70**. We first analysed the data from days 21-70 to see if the reported "masculinization" phenomena could be repeated, and followed this by looking at days 21-55 and 21-35 to further narrow (define) the period of any peripubertal window. By comparing various groups, we could subsequently examine the effects of testicular secretions on the defeminization and/or masculinization of P-450 2C11 and 3A2 activities during these time frames. Cytochrome P-450 2E1 activity will be measured by the formation of 4-hydroxyaminophenol from hydroxyaniline in defined incubation systems. P-450 2E1 is apparently not neonatally defeminized or regulated in the same manner as 2C11 or 3A2, and thus will act as a control. The activity of the female-specific P-450 2A1 was also measured in every group of animals. The activity was determined by the 7α-hydroxylation of testosterone.

2.0 GENERAL METHODS

2.1. Measuring P-450 specific activity

Differences between males and females can easily be measured by determining the specific activity of a number of enzymatic reactions, employing a number of different substrates. As mentioned earlier, specific activity measures the amount (nmol) of product formed in a specific reaction system, per minute, per milligram of crude microsomal protein (measured as nmol/min/mg). For general purposes, the measured velocity (*i.*e. specific activity) of a sex-specific P-450 isozyme will either represent a "masculine" value or a "feminine" value.

We will be monitoring the amount of cytochromes P-450 2C11, 3A2, 2A1 and 2E1 by measuring the specific activity of the individual isozymes, using the specified reactions (2α-, 16α-, 6β-, 7α-hydroxylations, ethylmorphine demethylation, aniline hydroxylation) as markers. We will also be measuring the total amount of P-450 present in our individual microsomal samples, as well as monitoring the specific activity of cytochrome *c* reductase (a cofactor in the P-450 reaction) to make sure that any apparent change in isozyme specific activity is not due to a change in total P-4-50 content or reductase activity.

The purpose of our study is to pharmacologically and/or surgically manipulate the endocrine status of developing male rats in such a way as to prevent the *neonatal* defeminization of certain sex-specific P-450s (2C11 and 3A2). We will then try to

bring about the same, or some, degree of defeminization/masculinization of these enzymes during a *different* (*i.e.* peripubertal) time-frame. We studied the hydroxylation of testosterone at the 2α-, 16α-, and 6β-positions, as well as the *N*-demethylation of ethylmorphine, which are reactions catalysed by P-450s 2C11 and 3A2, and are specific to the *male* rat. These end-points were chosen as proven sex-specific markers, which will tell us whether the sex-specific P-450 is present or not present, and will also give us a relative indication of the working speed of the individual enzyme system. The results can be correlated with literature values as well as our own control values. We also studied the 7α-hydroxylation of testosterone (*female*-specific) and the *p*-hydroxylation of aniline as controls, representing P-450s which are not defeminized and/or regulated in the same way as the male-specific P-450s.

2.2. Individual treatments and groups

We initially began by effectively "removing" the entire neonatal "critical" period (day 0-21) by blocking the aromatization of testosterone to estradiol in intact male rats, thus hoping to block neonatal defeminization. The aforementioned results from other laboratories (**section 1.3.2.**) indicated that ATD would be an excellent choice to elicit the required effect. Once the *neonatal* defeminization of P-450s 2C11 and/or 3A2 were blocked, we then sought to determine the masculinization of these specific P-450 enzymes as a function of having the testes present, at different time frames during this pubertal window.

Table 2.2.A. Experimental protocol for Sections One through Four.

Group	Day 0	Day 21	Day 35	Day 55	Day 70	Day 84
empty/21	implant empty capsule	remove capsule/ castrate			kill	
ATD/21	implant ATD	remove capsule/ castrate			kill	
ATD/21/T	implant ATD	remove capsule/ castrate			begin testosterone therapy	kill
ATD/35	implant ATD	remove capsule	castrate		kill	
ATD/55	implant ATD	remove capsule		castrate	kill	
ATD/55/T	implant ATD	remove capsule		castrate	begin testosterone therapy	kill
ATD/70	implant ATD	remove capsule			castrate	kill
ATD/intact	implant ATD	remove capsule			kill	

Table 2.2.B. Individual treatments for experimental groups of rats.

Group	Treatment
intact female/male	No treatment; killed day 70-90.
empty/21	Empty subcutaneous Silastic™ capsule day 0-21; castrated day 21; killed day 76-79.
ATD/21	Subcutaneous Silastic™ capsule containing ATD day 0-21; castrated day 21; killed day 76-79.
ATD/21/T	Subcutaneous Silastic™ capsule containing ATD day 0-21; castrated day 21; daily testosterone therapy day 70-83 (2mg/kg, injected s.c.); killed day 84.
ATD/35	Subcutaneous Silastic™ capsule containing ATD day 0-21; castrated day 35; killed day 70.
ATD/55	Subcutaneous Silastic™ capsule containing ATD day 0-21; castrated day 55; killed day 70.
ATD/55/T	Subcutaneous Silastic™ capsule containing ATD day 0-21; castrated day 55; daily testosterone therapy day 70-83 (2mg/kg, injected s.c.); killed day 84.
ATD/70	Subcutaneous Silastic™ capsule containing ATD day 0-21; castrated day 70; killed day 84 or 86.
ATD/intact	Subcutaneous Silastic™ capsule containing ATD day 0-21; killed day 70.
adult castrate	Castrated in adulthood (day 70-90); killed fifteen days later.

2.3. Section one

"Does ATD block neonatal defeminization?"

Within the first 16 hours of life, male pups were implanted with either ATD-filled capsules or empty sealed capsules. The ATD was delivered subcutaneously to the pups in a 6mm long Silastic™ capsule (Medical Grade tubing, Dow Corning, Michigan, U.S.A.), of interior diameter 1.56 mm and outer diameter 3.15 mm. The capsule was filled with 4 millimetres of ATD (as measured in capsule length), and sealed with Silastic™ Medical Adhesive (Silicone type A, Dow Corning, Michigan, U.S.A.). Numerous studies have shown subcutaneous Silastic™ capsules filled with crystalline ATD to be an effective delivery system for this compound in the neonatal rat (Swaab *et al.*, 1995; Vreeburg *et al.*, 1977; Ulibarri and Micevych, 1993; Bakker, 1995). Vreeburg *et al.* (1977) provide data that the ATD plasma concentration reaches 520±70 ng/ml in male neonatal rats given ATD in the above manner, and demonstrated that ATD administration does not affect normal circulating adult levels of testosterone.

On day 21 of life, the capsules were removed and both groups of animals were castrated (**section 3.2.**) to remove any source of testicular sex steroids that may, by virtue of being present during the peripubertal time frame, obscure any inhibition of neonatal defeminization. Animals who had received ATD-filled capsules were further divided into two groups, *i.e.* those that received testosterone during adulthood (2 mg/kg/day, day 70-83), and those that did not. The purpose of giving some of

these animals testosterone during adulthood was to see if the P-450 enzymes in question were subsequently responsive to testosterone. An increase in enzyme activity following exposure to testosterone could be indicative of neonatal programming (defeminization), or it could be due to an acute effect strictly from the presence of testosterone (masculinization). Animals were sacrificed on either day 70 (*ATD/21*) or day 84 (*ATD/21/ T*).

2.4 Section two

"Does the lifelong presence of the testes in the non-neonatally-defeminized male rat result in P-450 3A2 and 2C11 activity levels equal to those of the intact male?"

Within the first 16 hours of life, male pups were implanted with ATD-filled capsules for a period of 21 days, at which time the capsules were removed. On day 70 (*i.e.* early adulthood), half of animals (*ATD/intact*) were sacrificed, and the remaining animals (*ATD/70*) were castrated. The enzymes of the castrated animals were therefore not under the influence of any testicular steroids upon sacrifice of the animal. This will help to indicate whether or not any defeminization and/or masculinization that occurred between days 35-70 was a *permanent* event, and will also indicate whether any *basal* enzyme level was defeminized during that time frame. The *ATD/70* group was sacrificed on day 84.

2.5. Section three

"Peripubertal defeminization or acute masculinization?"

This experiment utilizes most of the experimental groups of animals. Comparisons will be made between animals castrated on various days (*ATD/21, ATD/35, ATD/55, ATD/70*), as well as between animals free of testicular hormones at the time of sacrifice (aforementioned plus *adult castrate*), and those receiving testosterone therapy (*ATD/21/T, ATD/55/T, ATD/intact*). We will be determining if any permanent defeminization of basal enzyme activities occurs, and we will also be determining if the enzymes are responsive to testosterone therapy in adulthood (two indications of defeminization).

2.6. Section four

"What length of time do the testes have to remain *in situ* for this peripubertal event to occur?"

2.6.1. Investigation of the presence of the testes during days 21-55: Within the first 16 hours of life, male pups were implanted with ATD-filled capsules for a period of 21 days, at which time the capsules were removed. On day 55 (*i.e.* mid-point of puberty), all animals were castrated and divided into two groups. On day 70, the *ATD/55* group was sacrificed and the remaining animals (*ATD/55/T*) received testosterone supplementation for a period of fourteen days. As the presence of the testes from day 21-70 did effectively masculinize P450s 2C11 and 3A2 in preliminary

studies, castrating the animals at day 55 will help to characterize the size of this peripubertal window. Any difference found between the specific activity of either 3A2 or 2C11 in each aforementioned group (those receiving testosterone and those not) would indicate whether or not this enzyme was responsive to testosterone (*i.e.* defeminized). There also exists the possibility that the rats may not be defeminized per se, but may acutely respond to the presence of testosterone in adulthood.

2.6.2 Investigation of the presence of the testes during days 21-35: Within the first 16 hours of life, male pups were each implanted with an ATD-filled capsule for a period of 21 days, at which time the capsule was removed. With neonatal defeminization blocked by the ATD, the testes remained *in situ* for a period of fourteen days (until day 35, *i.e.* the beginning of puberty), at which time the animals were castrated to remove testicular sex steroids. These animals were not exposed to testosterone at any point before sacrifice. It would have been ideal to have a group of animals castrated at day 35 and given testosterone from days 70-84, however due to a minimal availability of neonates we had to exclude such a group. The above group will be referred to as *ATD/35*.

2.7. Other animals

Adult males (age 70-80 days) were obtained from the Vivarium (Memorial University, St. John's, Newfoundland) and castrated during adulthood. This group of animals (*adult castrates*) represent defeminized animals. These animals have had intact testes from day 0 (birth) up to and including adulthood (neonatally defeminized and then masculinized) and then were demasculinized by removing the testes, and killed fourteen days later. The *male intact* and *female intact* groups (adult males and females) were obtained from the Vivarium and sacrificed at 80-100 days of age.

3.0. MATERIALS and METHODS

3.1. Animals

Sprague-Dawley females in the late stages of pregnancy were obtained from the Vivarium (Memorial University, St. John's, Newfoundland) and housed in individual plastic cages (43×22×21cm) on hardwood chip bedding (BPI Inc., Ste. Hyacinthe, Quebec). Food (Agway Prolab Rat/Mouse/Hamster 3000, Agway Inc., New York) and tap water were provided *ad libitum*. Animals were kept on a 12 hour light/dark cycle (lights on at 8:00 a.m.) at a constant room temperature of 21°C (15% relative humidity), until parturition. When pregnant dams could not be obtained, litters with their dams were obtained from the Vivarium (St. John's, Newfoundland) within 16 hours of parturition. On the day of parturition (day 0), the pups were separated from their dam and their sexes determined. Each male then underwent surgery to implant one Silastic™ capsule between the scapulae (as described below) under hypothermic anesthesia. This was followed by a one-hour recovery period at which time the pups were returned to their dams and litter sizes adjusted to 10 pups when possible. At 21 days of age, all pups were weaned and underwent surgery for removal of the capsule and some animals were castrated, as described in **section 3.2.**. Pups were assigned to treatments across litters where possible (*i.e.* each litter was divided into two treatment groups), and were housed with three other members of

their treatment group until day 35, at which time they were housed in pairs to await further treatments. All animals were killed by decapitation between day 70 and 86.

3.2. Surgery and treatments

3.2.1. Neonatal surgery: Within the first 12-16 hours of life, the pup was wrapped in a damp piece of gauze bandage and placed in ice for 7-10 minutes to induce hypothermic anesthesia (Pfeiffer, 1936). The pup was then removed from the ice and placed dorsal side up on an operating surface, and the skin was wiped with 95% ethanol. A small sagittal incision was made in the skin of the midline of the head between the ears. The skin between the scapulae was then freed from the underlying tissues with a fine probe. One Silastic™ capsule was inserted with forceps subcutaneously into the intrascapular space. Some animals received empty, sealed capsules. The incision was closed with cyanoacrylate surgical glue.

Following surgery, the pup was left undisturbed until its body temperature returned to room temperature, as indicated by pinkish skin colour and increased activity. All pups were then placed in an incubator at 30-32°C, for at least one hour before replacement to their respective dams. When possible, litter sizes were adjusted to 10 pups with the females of the litter. This procedure minimized the probability of maternal neglect and ensured equal maternal treatment of each pup.

3.2.2. Removal of ATD capsule: On day 21 of life, the capsule was removed under light ether anesthesia. A small area of skin of the thoracic region of the back was shaved and wiped with 95% ethanol. An incision large enough to allow removal of the capsule was made longitudinally with a scalpel. The capsule was manually pushed toward the incision and removed with a pair of foreceps. The incision was then closed with one or two sutures of surgical thread. The animal was left undisturbed until it had recovered from the anesthetic, at which time it was returned to its cage.

3.2.3. Castration: Castration of the rats was performed at four different ages - juvenile (21 days), prepubertal (35 days), pubertal (55 days) and adult (70 days). The procedure for castration at each age was essentially the same, however at the age of 21 days the testes are still located inside the abdominal cavity, thus the procedure was modified slightly.

The rat was placed under light ether anesthesia and an area of the scrotum was shaved and wiped with 95% ethanol. A longitudinal incision was then made in the middle of the scrotum with a scalpel. In the case of the juvenile castrates, the undescended testes were then gently pushed through the inguinal canal, until they descended into the scrotal cavity. A small incision was then made through the tunica until a testis could be extruded to the exterior. A single ligature was placed around each of the internal spermatic artery and the venous complex. The testis, together with the epididymis, was then excised, and the remaining tissue was pushed back up

the inguinal canal into the abdomen with forceps. The same steps were followed for the other testis. The incision was closed with either one or two sutures and wiped with 95% ethanol. The animal was then left undisturbed until it had recovered from the anesthetic, at which time it was returned to its cage.

3.2.4. Testosterone supplementation: Some adult animals previously castrated at either day 21 (*ATD/21/T*) or day 55 (*ATD/55/T*) received daily injections of testosterone at a dosage of 2mg/kg, for a period of fourteen days beginning on day 70. The testosterone was dissolved in corn oil and delivered subcutaneously into the intrascapular space. It was injected via a 1.0 ml plastic syringe with a 27-gauge needle (5/8 inch in length), in a volume equal to 0.1% of the animal's body weight.

3.3. Buffer preparation

The buffers were prepared according to Gomori (1955). Tris-HCl (50 mM) pH 7.4 was used in most *in vitro* assays as well as for the final suspension of microsomal protein. Tris-HCl (50 mM) pH 7.4 containing 150 mM KCl was used in microsomal preparation. The cytochrome *c* reductase assay was carried out in 33 mM potassium phosphate (KP_i) buffer. All pH measurements were made using a Fisher Accumet Model 620 pH meter (Instrument Division, Fisher Scientific, Pittsburgh, Pennsylvania) equipped with a calomel sleeve junction reference electrode and a universal glass body indicating electrode, following a 2-point calibration.

Buffer preparation (continued)

50 mM Tris-HCl: Add 50 ml of stock 0.2 M Tris (24.2g in 1000 ml H_2O) to 41.4 ml of stock 0.2 M HCl (17.25 ml in 1000 ml H_2O) and dilute to 200 ml with distilled water. Adjust the pH to 7.400 with concentrated HCl or NaOH.

50 mM Tris-HCl (150 mM KCl): Add 11.2g KCl to 1000 ml 50 mM Tris-HCl.

100 mM KP_i: Add 13 ml stock 0.2 M monobasic KP_i (27.2g in 1000 ml H2O) to 87 ml stock 0.2 M dibasic KP_i (45.65 g in 1000 ml H_2O) and dilute to 200 ml with distilled water. Adjust the pH to 7.6. Since 33 mM KP_i was used in the cytochrome *c* reductase assay, simply dilute this stock solution one part solution to two parts distilled water.

3.4. Preparation of microsomes

The rat was decapitated and exsanguinated. The liver was rapidly excised and placed in ice-cold 50 mM Tris-HCl buffer containing 150 mM KCl, pH 7.4. The tissue was agitated with the use of forceps to remove hair, blood clots, etc. The liver was then blotted dry and weighed. The tissue was transferred to a beaker containing three volumes of fresh ice-cold Tris-KCl, and minced with small scissors. The beaker containing the liver was placed in a larger beaker holding ice-water, to maintain a constant temperature. The liver was then homogenized using a Brinkmann homogenizer (model PT 10/35, Brinkmann Instruments, Switzerland) in a cold room maintained at 4°C. The homogenization was effected by placing the cutting probe (120 mm outer diameter) at a 45° angle, and homogenizing the mince with two 15-second pulses separated by one minute, at setting 4.0. The homogenate

was then centrifuged at 1000 g for five minutes and at 10 000 g for 15 minutes in a (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Dupont Instruments, Delaware, U.S.A.). The chamber was pre-chilled to 4°C prior to use.

The supernatant was strained through three layers of cheesecloth into a centrifuge tube. Approximately 10-20 ml of this solution was divided equally into two separate ultracentrifuge tubes. These samples were further centrifuged at 105 000 g (L8-70M Ultracentrifuge, Beckman Instruments Inc., California, U.S.A.) for 80 minutes at 4°C. The supernatant was then discarded from both tubes, and each pellet was rinsed with 5 ml of 50 mM Tris-HCl buffer.

Unwashed microsomes: One of the microsomal pellets was then resuspended in 50 mM Tris-HCl buffer, pH 7.4, by use of a glass mortar and pestle. The volume was equal to 30-50% of the volume of the original sample of 10 000 g supernatant, giving a final protein concentration of 15-20 mg/ml. The protein concentration of this unwashed sample was then determined by the Bradford protein assay (Bradford, 1976) (**section 3.5.1.**). The ethylmorphine demethylase assay (**section 3.5.2.**) was then immediately performed on these samples.

Washed microsomes: The other microsomal pellet was rinsed with 5 ml of 50 mM Tris-HCl buffer prior to washing. Washing of the microsome sample was effected by resuspending the pellet (as described above) in a volume equal to that of the original 10 000 g supernatant. This suspension was then centrifuged at 105 000 g for 60 minutes (4°C) as described previously. The supernatant was then discarded and the pellet rinsed with 5 ml of 50mM Tris-HCl buffer. This step was

followed by a final resuspension in a volume of 50 mM Tris-HCl buffer equal to 30-50% of the volume of the original 10 000 g supernatant. This gave us a desired final protein concentration of 15-20 mg/ml. Glycerol was then added to a final concentration of 20% (v/v) to protect the microsomes from the detrimental effects of freezing and thawing. The protein concentration was determined by the Bradford protein assay, and the microsome suspensions were frozen at -80°C for future use.

3.5. Assays

3.5.1. Measurement of protein concentration: Microsomal protein concentrations were determined using the Bradford protein assay (Bradford, 1976), with bovine serum albumin dissolved in 50 mM Tris-HCl, pH 7.4 as the standard. The stock suspension of microsomes was diluted 1:2000 in 50 mM Tris-HCl, pH 7.4. To 800 µl of this solution, 200 µl of dye reagent was added, and the resultant solution was mixed and allowed to sit at room temperature for a minimum of 20 minutes. A blank consisting of 800 µl of buffer and 200 µl dye reagent was treated similarly. Duplicates of known protein concentrations of 2, 4, 6, 8 and 10 µl/ml were assayed simultaneously to produce a standard curve. Absorbancies of all samples and blanks were measured at 595 nm in a UV-Vis spectrophotometer (DU-70, Beckman Instruments Inc., California). Protein concentrations were estimated from the resultant standard curve (**Figure A.1.**).

3.5.2. Ethylmorphine N-demethylase assay: The N-demethylation of ethylmorphine was determined from the amount of formaldehyde produced using the Nash reaction as described by Cochin and Axelrod (1959). The incubate contained, in a total volume of 3.0 ml, 50 mM Tris-HCl (pH 7.4), 1.0 mM NADP, 3.3 mM glucose-6-phosphate, two units of glucose-6-phosphate dehydrogenase, 8.3 mM $MgCl_2$, 1.0 mM semicarbazide, 6.0 mg microsomal protein, and 7.0 mg of ethylmorphine hydrochloride.

The incubate, minus the microsomal protein, was agitated for five minutes in a Dubnoff metabolic shaking incubator (GCA Corporation, Chicago, Illinois), under air, in a 25ml Erlenmeyer flask at 37°C. The reaction was started by adding the microsomal protein to the incubation flask and the reaction was allowed to proceed for 15 minutes. Reaction rates were linear with respect to time and protein concentration under these conditions (see Appendix). Tissue blanks were run for each sample and contained no ethylmorphine hydrochloride in the initial incubation system. To terminate the reaction, the incubate was added to a 15 ml Corex tube containing 2.0 ml of 20% (w/v) $ZnSO_4$. To this mixture was added 2.0 ml of saturated $Ba(OH)_3$. All samples were then centrifuged (Beckman GP Centrifuge) for 10 minutes at 3000 g to precipitate the protein and the $Ba_2(SO_4)_3$. A 5 ml aliquot of the supernatant was then pipetted into another tube and 2.0 ml of double strength Nash reagent were added. All samples and blanks were agitated in a water bath (Haake SWB-20, Fisons, West Germany) at 60°C for 30 minutes. The absorbencies were then measured immediately at 413 nm, in Beckman UV-VIS spectrophotometer

and compared to the formaldehyde standard curve (**Figure A.2.**). The specific activity (S.A.) was determined as follows:

$$\text{nmol formaldehyde} = \left[(A_{413} - \text{blank}) - 0.03891\right] \div 0.0023935$$

$$\text{S.A.} = \text{nmol formaldehyde} \div \text{total incubation time (minutes)} \div \text{protein (mg)}$$

where A_{413} = absorbance at 413 nm

3.5.3. <u>NADPH cytochrome *c* reductase assay</u>: Microsomal NADPH cytochrome *c* reductase activity, expressed as nanomoles cytochrome *c* reduced per minute per milligram of microsomal protein, was determined spectrophotometrically at 550 nm. Incubation mixtures (3 ml) contained 33 mM KP_i, pH 7.6, 44 μM NADPH, 50 mM oxidized cytochrome *c*, 90 μg microsomal protein, and 1 mM KCN. The blank did not contain NADPH.

The incubation mixture minus the NADPH was prepared in a 5 ml cuvet. Following calibration of the Beckman UV-VIS spectrophotometer, the cuvet was placed in the holding cell and scanned for background interference at 550 nm. The NADPH was then added to the incubate and the reaction allowed to proceed for 4 minutes while the absorbance of the reduced cytochrome *c* was recorded at one and four minutes respectively, at a wavelength of 550 nm. The absorbance from 0 minutes to one minute was not measured, as the change in absorbance is not linear

during this time frame (preliminary lab results). Specific activity (S.A.) was determined as follows:

$$S.A. = \left[(A_2 - A_1) \div \varepsilon \times (\text{volume})(1000\text{ml.l}^{-1}) \right] \div (\Delta\text{time})(\text{mgMP})$$

where: A_1 = absorbance reading @ time=1.0 minutes
A_2 = absorbance reading @ time=4.0 minutes
ε = molar extinction coefficient @ $19.6\text{cm}^{-1}\text{mM}^{-1}$
MP = microsomal protein
volume = 3ml

Optimal conditions for microsomal-bound reductase activity were determined in another study (our laboratory), using liver microsomes prepared from intact adult male Sprague-Dawley rats (Memorial University, St. John's, Nfld., Canada). Optimal conditions were used to ensure that the reductase was operating under the conditions which gave optimal enzyme activity. The pH of each incubation system was varied between 7.5 and 8.0, and samples were run in triplicate in order to determine the optimal pH. To determine the optimal ionic strength for maximal specific activity, the same experiment was run at pH = 8.0 and the ionic strength of the KP_i buffer was varied from 0.04 to 0.94 by adding increments of KCl. The optimal NADPH concentration was determined by varying the amount of NADPH in the incubation system (0.17 μM to 44.4 μM) while keeping the concentration of cytochrome *c* constant at 50 μM. The subsequent specific activity was plotted *vs.* [NADPH], and the K_m and V_{max} determined by Eadie-Hofstee plots. The optimal concentration of cytochrome *c* was determined by varying the amount of cytochrome *c* in the

incubation system (0.62 μM to 158 μM) while keeping the concentration of NADPH constant at 44 μM. The subsequent specific activity was plotted *vs.* [cytochrome *c*], and the K_m and V_{max} determined by Eadie-Hofstee plots. The concentrations of cytochrome *c* and NADPH were determined by Eadie-Hofstee plots to be enzyme saturating.

3.5.4. <u>Cytochrome P-450 assay</u>: Total hepatic microsomal cytochrome P-450 content was determined from the sodium dithionite-reduced carbon monoxide difference spectrum using a molar extinction coefficient of 91 cm^{-1} mM^{-1} (Omura and Sato, 1964). Microsomal suspensions of 1-2 mg/ml were added to a cuvet, to which were added a few milligrams of sodium dithionite. The Beckman UV-VIS spectrophotometer was calibrated with this suspension. Carbon monoxide was gently bubbled into this suspension for 30 seconds, and the absorbance of the resulting suspension was then measured between 400 and 490 nm. The total P-450 per mg microsomal protein (MP) was determined by the following equation:

$$\text{X } \mu\text{mol P-450/ml} = \left[(A_{450} - A_{490}) \div \varepsilon\right] \times 1000\mu\text{mol/mol}$$

$$\text{nmol P-450/mg MP} = 1000\text{nmol}/\mu\text{mol} \times \text{X } \mu\text{mol P-450/ml} \div \text{microsomal protein/ml}$$

where: ε = molar extinction coefficient of 91 $cm^{-1}mM^{-1}$
A_{450} = absorbance at 450nm
A_{490} = absorbance at 490nm

3.5.5. Aniline hydroxylase assay: The hydroxylation of aniline was determined from the amount of hydroxyaniline (4-aminophenol) produced by 3 ml incubation systems containing 50 mM Tris-HCl (pH 7.4), 1.0 mM NADP, 3.3 mM glucose-*6*-phosphate, two units glucose-*6*-phosphate dehydrogenase, 3.0 mg microsomal protein, 8.3 mM magnesium chloride and 1.1 µl aniline.

The incubate minus the microsomal protein, was agitated for five minutes in an incubator, under air, in a 25 ml Erlenmayer flask at 37°C. The reaction was started by adding the microsomal protein to the incubation flask and the reaction was allowed to proceed for 20 minutes. Reaction rates were previously determined to be linear with respect to time and protein concentration. To terminate the reaction, the incubate was added to a 15 ml Corex tube containing 1.5 ml of 20% trichloroacetic acid (w/v). All samples were then centrifuged (Beckman GP centrifuge) for 10 minutes at a setting of 2000 g. A 3 ml aliquot of the supernatant was then pipetted into another tube and 1.5 ml of 10% Na_2CO_3 (w/v) and 3 ml of 0.2 M NaOH containing 2% phenol (w/v) was then added. The absorbance of each sample was then measured after full colour development (predetermined to be 25 minutes) at 630 nm and compared to the hydroxyaniline standard curve (**Figure A.3.**).

3.5.6. Testosterone assay: The hydroxylations of testosterone at the 2α- 16α- , 6β- and 7α- positions were determined from the amount of individual metabolites produced by 1.000 ml incubation systems containing 0.020 ml testosterone (3.6 mg/ml of methanol), 0.180 ml of Tris buffer (50 mM, pH 7.4), 0.200

ml sucrose (85 mg/ml of Tris), 0.050 ml of $MgCl_2$ (12 mg MgCl2•6HOH/ml of water), 0.050 ml NADPH solution (1 mg/ml Tris) and 0.500 ml microsomal protein (2 mg/ml Tris).

The incubate minus the NADPH was agitated for 5 minutes in an incubator, under air, in a 5 ml Corex test tube at 37C. The reaction was started by adding the NADPH solution to the incubate, and allowed to react for exactly 10 minutes. The reaction was terminated by adding 6 ml of dichloromethane to the incubate. This solution was then vortexed for 15 seconds and centrifuged (Beckman GP centrifuge) for 5 minutes at 3000 g. Five millilitres of the organic phase was then pipetted to a conical tube and evaporated to dryness under a gentle stream of N_2 gas.

The residue in the conical tube was the dissolved in 100 ml of solution A (containing an internal standard) and transferred to a sealed vial. The samples were then ready to be tested.

The chromatography system (Beckman, Toronto, Ontario) consisted of two 11B Solvent Delivery Modules, a 507 Autosampler, a 406 Interface Module and a 116 Programmable Detector. The system is computer controlled using Beckman System Gold software. The column, a 150 mm 4.6 mm ID Supelcosil LC18 (Supelco Inc., Toronto, Ontario), was preceded by a 20 mm 4.6 mm ID guard column of octyldecylsilane (Supelco Inc., Toronto, Ontario) and a 2 mm inlet filter (Rheodyne, Inc. Coati, CA). The column was eluted for 30 minutes, at a flow rate of 1.5 ml per minute, with a concave gradient (Programme 4 of the Interface Module) beginning at 100% Solvent A (43% aqueous methanol containing 1.1% acetonitrile)

and finishing with 100% Solvent B (75% aqueous methanol containing 1.9% acetonitrile). The identity of unknown peaks was established by comparing their retention times, relative to androstenedione (the internal standard) (RT = 22.1 0.3 minutes), with the ratios for known standards. Quantification of metabolites was done by comparing their peak areas with plots of mass *vs.* peak area for known standards (for calibration curves, see **Figures A.4. - A.7.**).

3.6. Statistical Analyses

To determine the significance of drug effects on the biological parameters the data were subjected to the student *t*-test using the SigmaStat 2.0 program. Data were analyzed in four different groups, representing Results Sections One through Four, and the *t*-test was performed on every paired comparison, at $p<0.05$ and $p<0.10$. Ideally, four different experiments would be run, each with its own set of controls. Due to time restraints, we ran one experiment, and analyzed the data four ways.

4.0. RESULTS

4.1. Section one

"Does ATD block neonatal defeminization?"

The initial experiment was carried to see if ATD delivered subcutaneously in Silastic™ capsules from day 0-21 was effective in blocking the neonatal defeminization of P450 2C11 and/or 3A2. Neonatal defeminization may be seen as a permanent increase in basal enzyme specific activity as compared with the intact female. As well, defeminization may also be characterized as *a* reversible *responsiveness* of the enzyme to testosterone in adulthood. In other words, a non-defeminized male will *not* respond to testosterone in adulthood, whereas a defeminized male will respond (shown by an increase in enzyme specific activity due to the presence of testosterone).

We will be comparing the following groups of animals: *empty/21*, *ATD/21* and *ATD/21 T* against each other as well as against the control groups (*intact male*, *intact female*, *adult castrate*). The *empty/21* represents neonatally defeminized males who have been exposed to testicular factors only from day 0-21, and are subsequently castrated and sacrificed in adulthood. They will be compared to the *intact male* and *intact female* in order to determine if neonatal defeminization is characterized by an increase in basal (absence of testosterone) P-450 velocities. The *adult castrate* male represents neonatally defeminized males who have been exposed to testosterone from

day 0-70. There may or may not exist a difference between the two neonatally defeminized groups (*empty/21*, *adult castrate*).

Both neonatally defeminized groups will also be compared to *ATD/21*. The *ATD/21* presumably represent non-defeminized males who have not had their testes removed until day 21, but have not been exposed to the neonatal effects of testosterone (*i.e.* estradiol) during the "critical" neonatal period. Specific activities obtained from the *ATD/ 21* group that are not significantly different from *intact females* may indicate that neonatal defeminization has been prevented. On the other hand, this may also mean that neonatal defeminization has occurred, but is not represented by a permanent increase in basal enzyme velocity because of the absence of some factor in the male.

Since neonatal defeminization is often represented, in part, as an increased responsiveness of adult P-450 to circulating testosterone, the *ATD/21/T* group would provide information as to whether or not the specific P-450s could be "activated" in adulthood, and to what extent.

4.1.1. P-450 3A2: Ethylmorphine demethylase (Table 4.6.A):

Sex differences, characterization of defeminization: The specific activity of EMDM showed the expected sex differences with *intact male* (7.2 ± 0.47) being statistically greater ($p<0.05$) than *intact female* (1.4 ± 0.11). The neonatally defeminized group *adult castrate* (2.2 ± 0.15) was statistically greater ($p<0.05$) than the *intact female*, demonstrating that neonatal defeminization of EMDM is

characterized as an increase in basal enzyme specific activity *greater* than that of the female. On the other hand, the other group of defeminized males (*empty/21*) had EMDM specific activities (2.9 ± 1.14) that were not statistically different ($p<0.05$) from that of the *intact females* (1.4 ± 0.11). Even though the defeminized EMDM velocity was 2-fold greater than the *intact female*, there was a large variation in individual animal EMDM velocities (standard deviation was ± 2.56), which led to the large standard error shown.

The effect of ATD from day 0-21: When the defeminized *empty/21* or *adult castrate* is compared with the alleged non-defeminized *ATD/21*, we do not see a significant change in EMDM specific activity. The *ATD/21* EMDM velocities were not significantly different from the *intact female* (1.7 ± 0.19 *vs.* 1.4 ± 0.11; $p<0.05$). These observations may or may not support the conclusion that neonatal defeminization had been blocked by neonatal ATD treatment, larger *n* values would help clarify this concept.

Evidence that ATD blocked neonatal defeminization is presented in the data from the groups of animals who were neonatally treated with ATD and were castrated at some point prior to adulthood (*ATD/55* and *ATD/70*). The *adult castrate* shows that the velocity of EMDM will drop to a female value upon removal of testicular factors, *i.e.* in a neonatally defeminized but not masculinized male. What we found in the *ATD/55* and *ATD/70* is the velocity of EMDM drops to a value statistically *higher* than that of the *adult castrate*. If these animals had been neonatally defeminized, it is logical that their basal enzyme velocity upon castration would not

be different than that of the *adult castrate*. This is because they would be essentially the same animal endocrinologically, the only difference being the absence of estradiol from day 0-21.

The effect of testosterone in adulthood: The exposure of the *ATD/21* (1.7 ± 0.19) to testosterone in adulthood (*ATD/21/T*; 4.8 ± 0.42) resulted in statistically significant ($p<0.05$) 2.7-fold increase in the specific activity of EMDM. Unfortunately, this suggests that either neonatal defeminization *had* occurred in the presence of ATD (and the enzymes are thus responding to circulating testosterone), or that the enzyme is responding to circulating testosterone in the *absence* of neonatal defeminization. The latter explanation seems more reasonable as the specific activity of EMDM in the *ATD/21/T* group was statistically less ($p<0.05$) than both the *intact male* and the *ATD/intact* activity. The literature suggests that a neonatally defeminized male will respond to testosterone in adulthood, with activities reaching *full intact male values* (El Defrawy El Masry and Mannering, 1974; Kramer *et al.*, 1975b).

4.1.2. P-450 3A2: 6β-hydroxylase (Table 4.6.B):

Sex differences, characterization of defeminization: The specific activity of 6β-hydroxylase showed the expected sex differences with *intact male* (0.28 ± 0.062) being statistically greater ($p<0.05$) than *intact female* (0.06 ± 0.035). The neonatally defeminized *adult castrate* (0.13 ± 0.010) and *empty/21* (0.18 ± 0.086) were not statistically different from either the *intact male* (0.28 ± 0.062) or the *intact*

female (0.06 ± 0.035). Thus it is difficult to say whether or not neonatal defeminization is represented by an increase in basal enzyme specific activity to values greater than the female.

The effect of ATD from day 0-21: To evaluate the effects of ATD on the neonatal defeminization process, we compared the *empty/21* to the "non-defeminized" *ATD/21*. We found that statistically there was no difference between the two groups, although the *empty capsule/21* (0.18 ± 0.086) was 89-fold greater than the *ATD/21* (0.002 ± n.d.). It is likely that the large standard deviation within the *empty/21* group (0.18 ± 0.172) contributed to the difficulty in characterizing a statistical difference between these two groups. The velocity in the *ATD/21* (0.002 ± n.d.) was not statistically different ($p<0.05$) from the *intact female* (0.06 ± 0.035), however it was significantly *less* ($p<0.05$) than the *intact male* (0.28 ± 0.062), the *adult castrate* (0.13 ± 0.010) and the *ATD/21/T* (0.34 ± 0.030).

The effect of testosterone in adulthood: The exposure of the *ATD/21* (0.002 ± n.d.) to testosterone in adulthood ATD/21/T (0.34 ± 0.030) resulted in a statistically significant ($p<0.05$) *increase* in the velocity of 6β-hydroxylase. Unlike EMDM, the presence of testosterone was sufficient to *fully* masculinize 6β-hydroxylase velocity to *intact male* values (0.28 ± 0.062).

4.1.3. P-450 2C11: 2α-hydroxylase (Table 4.6.C):

Sex differences, characterization of defeminization: The amount of P-450 2C11 can be estimated by measuring its specific activity. The specific activity of P-450 2C11 can be measured by the quantitating the hydroxylation of testosterone at the 16α- (selective) and 2α- (specific) positions.

The specific activity of 2α-hydroxylase showed the expected sex differences with the *intact male* (1.66 ± 0.170) being statistically greater ($p<0.05$) than that of the *intact female* (0.24 ± 0.044). The neonatally defeminized males (*empty/21* and *adult castrate*) gave 2α-hydroxylase activities (1.08 ± 0.468 and 1.00 ± 0.028 respectively) that were approximately 4.5-fold greater than the *intact female* (0.24 ± 0.044). Of the two defeminized groups, only the *adult castrate* was statistically significantly greater than that of the *intact female*. Again, due to the large standard deviation in the *empty/21* group, it is difficult to carry out accurate statistics with this group. The defeminized adult castrate (1.00 ± 0.028) is only 65% of the *intact male* (1.66 ± 0.17) which is significant at $p<0.10$. It appears as though the defeminized *adult castrate* does not belong to either the *intact female* or the *intact male* group, suggesting that defeminization is seen as a permanent increase in basal enzyme velocity to values greater than that of the female, but not reaching male values.

The effect of ATD from day 0-21: To evaluate the effects of neonatal ATD exposure on neonatal defeminization, we compare the *ATD/21* group to the defeminized groups (*empty/21* and *adult castrate*). We found that the specific activity in the *ATD/21* (0.32 ± 0.125) was 68% less than the *adult castrate* (1.00 ±

0.028) ($p<0.05$) and 71% less than the *empty/21* (1.08 ± 0.468). Again, due to the large standard deviation in the *empty/21* (1.08 ± 0.935) and the small sample size (4), we cannot say that the 71% decrease was statistically significant. The velocity of the *ATD/21* was not statistically different ($p<0.05$) than the *intact female* (0.24 ± 0.044), reaffirming that neonatal ATD treatment was effective in blocking neonatal defeminization of 2α-hydroxylase since defeminization is seen as a permanent increase in basal enzyme velocity to values *greater* than that of the female.

The effect of testosterone in adulthood: The specific activity of 2α-hydroxylase increased significantly ($p<0.05$) in ATD-treated males castrated on day 21 (0.32 ± 0.125) and subsequently treated with testosterone in adulthood (*ATD/21/T*; 1.44 ± 0.067). The specific activity increased 4.5-fold to 1.44 (± 0.067), which is not statistically different ($p<0.05$) than the *intact male* (1.66 ± 0.170). This is suggestive of a complete "masculinization" response to circulating testosterone to values not different from the intact male, since neonatal and/or peripubertal imprinting should not have occurred.

4.1.4. P-450 2C11: 16α-hydroxylase (Table 4.6.D):

Sex differences, characterization of defeminization: The specific activity of 16α-hydroxylase showed the expected sex differences with the *intact male* (0.98 ± 0.149) being statistically greater ($p<0.05$) than the *intact female* (0.15 ± 0.044). The neonatally defeminized males (*empty/21* and *adult castrate*) gave 16α-hydroxylase activities (0.68 ± 0.315 and 0.48 ± 0.039 respectively) that were greater than the

intact female (0.15 ± 0.044), however only the adult castrate was statistically significantly greater ($p<0.05$). Because of the large standard deviation in the *empty/21* group, it is difficult to say whether it belongs to the *intact female* group or the *intact male* group. The *adult castrate* on the other hand, is significantly greater ($p<0.05$) than the *intact female* and is statistically less ($p<0.10$) than the *intact male* group. Because this group of animals represents neonatally defeminized males, these results indicate that neonatal defeminization can be seen as an increase in uninduced 16α-hydroxylase velocity.

The effect of ATD from day 0-21: To evaluate the effects of neonatal ATD exposure on neonatal defeminization, we compared the *ATD/21* to the defeminized groups (*empty/21* and *adult castrate*). The specific activity of the *ATD/21* (0.16 ± 0.040) was 66% less ($p<0.05$) than the *adult castrate* (0.48 ± 0.039) and 76% less than the *empty/21* (0.68 ± 0.315) (not significant due to large standard error). The velocity of the *ATD/21* was not statistically different ($p<0.05$) than the *intact female* (0.15 ± 0.044), reaffirming that neonatal ATD treatment was effective in blocking neonatal defeminization of 16α-hydroxylase.

The effect of testosterone in adulthood: Similar to 2α-hydroxylase, the velocity of 16α-hydroxylase increased significantly ($p<0.05$) in ATD-treated males castrated on day 21 (0.16 ± 0.040) and subsequently treated with testosterone in adulthood (*ATD/21/T*; 0.88 ± 0.032). The specific activity increased 5.5-fold to 0.88 ± 0.032, which is not statistically different ($p<0.05$) from the *intact male* (0.98 ± 0.149). This is suggestive of an acute "masculinization" response to circulating

testosterone to intact male values, since neonatal defeminization and/or peripubertal imprinting should not have occurred.

4.2. Section two

"Does the lifelong presence of the testes in the non-neonatally-defeminized male rat result in P-450 3A2 and 2C11 activity levels equal to those of the intact-defeminized male?"

4.2.1. P-450 3A2: Ethylmorphine demethylase and 6β-hydroxylase (Tables 4.6.A and 4.6.B): EMDM activity levels in the *ATD/intact* group (8.2 ± 0.59) were not significantly different from the *intact male* group (7.2 ± 0.47), thus indicating that the presence of the testes throughout the life of the non-neonatally defeminized animal results in a completely comparable "masculinization" of the 3A2 enzyme. This concept is further supported by the increase in specific activity of testosterone 6β-hydroxylase in these same two groups, *i.e.* the *ATD/intact* has a velocity of 0.26 (± 0.143) which is not significantly different ($p<0.05$) than that of the *intact male* whose velocity is 0.28 ± 0.062. Thus, the lifelong presence of the testes in the non-defeminized male will result in full 100% masculinized velocity of P-450 3A2.

4.2.2. P-450 2C11: Testosterone 16α- and 2α-hydroxylase (Tables 4.6.B. and 4.6.C.): The activity of 16α-hydroxylase in the *ATD intact* group (1.08 ± 0.105) was not statistically different from that of the *intact males* (0.98 ± 0.149), thus indicating that the presence of the testes throughout the life of a non-neonatally defeminized male will result in a complete masculinization of 16α-hydroxylase. The activity of 2α-hydroxylase was also completely masculinized in the non-neonatally defeminized male due to the presence of intact testes from day 21-70. Our data show that the *ATD intact* (1.89 ± 0.015) was not statistically different from the *intact male* (1.66 ± 0.170). Thus, the lifelong presence of the testes in the non-defeminized male will result in a 100% masculinized velocity of P-450 2C11.

4.3. Section three

"Peripubertal defeminization or acute masculinization?"

We conducted this experiment to see whether or not the peripubertal presence of the intact testes defeminizes 3A2 and/or 2C11 in the same way (*i.e.* by characteristic changes in specific activities) that the neonatal presence of the testes does.

The basal activity levels of 3A2 and 2C11 will be determined in males castrated at various peripubertal ages (day 21,35, 55, or 70) to determine if a permanent defeminization of an increase in basal enzyme velocities occurs. We also wanted to determine if testosterone was the sole necessary factor to evoke any

defeminization or masculinization, or if other testicular secretions are required or can contribute as factors. The adult responsiveness of 3A2 and 2C11 to testosterone will be characterized in the *ATD/21/T*, *ATD/55/T* and *ATD/intact* groups as they are compared to their respective control groups.

4.3.1. P-450 3A2: Ethylmorphine demethylase (Table 4.6.A):

Defeminization of a basal specific activity: A peripubertal defeminization process which lasted at least until day 84 of life, can be seen by the significant ($p<0.05$) 1.9-fold increase in basal EMDM specific activity imprinted from day 35 (2.1 ± 0.21) to day 55 (4.0 ± 0.14) in non-defeminized animals. No apparent increase in basal EMDM velocity occurred between day 21 (1.7 ± 0.19) and day 35 (2.1 ± 0.21), as there was no significant ($p<0.05$) increase in specific activity. The *ATD/70* (3.9 ± 0.50) was not significantly ($p<0.05$) different than the *ATD 55* (4.0 ± 0.14) indicating that the extra 15 days that the testes remained *in situ* did not have any further imprinting effect on EMDM. Therefore, this peripubertal imprinting appears to be a permanent event which is completed by day 55.

In Section One, we defined neonatal defeminization as an *increase in basal enzyme activity*, over and above that of the *intact female* (*adult castrate* vs. *intact female*). Thus, peripubertal defeminization appears to be similar to neonatal defeminization in that regard. However, the activity of EMDM increases 3-fold over that of the *intact female* in the peripubertally defeminized animals castrated at day 55 and/or day 70, and this increase is 2-fold ($p<0.05$) *greater* than the neonatally

defeminized *adult castrate*. It appears that EMDM basal enzyme activity can be permanently defeminized during puberty to activity levels higher than those attained due to neonatal defeminization.

Response to testosterone in adulthood: The relative responsiveness of EMDM to circulating testosterone in adulthood can be compared between animals castrated on day 21 and those castrated on day 55. Non-defeminized males (*ATD/21*) respond to testosterone with a 2.8-fold increase ($p<0.05$) in specific activity, whereas males castrated on day 55 only respond by an added 1.4-fold increase ($p<0.10$) in activity. The EMDM enzyme in the *ATD/21/T* is apparently not operating at its maximal specific activity, due to the fact that *ATD/21/T* (4.8 ± 0.42) is statistically less ($p<0.05$) than the *ATD/intact* (8.2 ± 0.59). This suggests that either neonatal defeminization is necessary for complete adult responsiveness of EMDM to testosterone, or that other testicular factors are needed to elicit the full adult masculinization. In the absence of neonatal or peripubertal defeminization, testosterone appears to be sufficient to cause at least a partial masculinization of EMDM, as there was a significant difference between the *ATD/21* (1.7 ± 0.19) and *ATD/21/T* (4.8 ± 0.42) groups. However, because the *ATD/21/T* group was significantly less than the *intact male* (7.2 ± 0.47), this leads us to believe that it may *not* be possible to fully masculinize EMDM in the absence of neonatal or peripubertal defeminization.

The *ATD/55/T* (5.7 ± 0.79) demonstrated an EMDM specific activity that was not significantly different from the *intact male* (7.2 ± 0.47), which indicates that

testosterone is a sufficient testicular hormone required for the full peripubertal masculinization effect in non-neonatally-defeminized males.

4.3.2. P-450 3A2: 6β-hydroxylase (Table 4.6.B.):

Defeminization of a basal specific activity: 6β-hydroxylase specific activity increased 55-fold when the testes were *in situ* from day 21-35 (0.002 ± n.d. to 0.11 ± 0.042). Whether or not this is a significant increase is not clear, as $p = 0.082$ which is very close to significance. However, 6β-hydroxylase specific activity increased 2.4-fold ($p<0.05$) if the testes remained *in situ* from day 35-55 (from 0.11 ± 0.042 to 0.27 ± 0.022). The basal specific activity of 6β-hydroxylase dropped back to day 35 levels (0.11 ± 0.042) however, if the testes remained *in situ* from day 21-*70* (0.07 ± 0.055).

Response to testosterone in adulthood: Circulating testosterone in adulthood is effective in restoring 6β-hydroxylase specific activity to intact adult male levels whether the non-defeminized male was castrated at age 21, 55 or 70 days. There were no statistically significant differences between the *ATD/21/T* (0.34 ± 0.030), *ATD/55/T* (0.31 ± 0.046), *ATD intact* (0.26 ± 0.143) and the *intact male* (0.28 ± 0.062). This suggests that the high degree of responsiveness of 6β-hydroxylase to circulating testosterone in adulthood is an *acute* event (*i.e.* non-permanent), and does not require defeminization to be effected.

4.3.3. P-450 2C11: 2α-hydroxylase (Table 4.6.C.):

Defeminization of a basal specific activity: A permanent peripubertal defeminization of 2α-hydroxylase is shown by the 2.4-fold increase ($p<0.05$) in the basal enzyme velocity, effected by the presence of intact testes from day 35 (0.55 ± 0.192) to day 55 (1.32 ± 0.057). No significant increase is seen in the specific activity of 2α-hydroxylase in non-neonatally-defeminized males if the testes are *in situ* from day 21 (0.32 ± 0.125) to day 35 (0.55 ± 0.192). The presence of the testes from day 55-70 in non-defeminized males appears to lead to a significant *drop* in 2α-hydroxylase specific activity, from 1.32 ± 0.057 to 0.96 ± 0.092.

Response to testosterone in adulthood: A significant 1.5-fold increase ($p<0.05$) in specific activity of 2α-hydroxylase (from 1.32 ± 0.057 to 1.96 ± 0.165) is seen, due to adult exposure to testosterone from day 70-83 in these peripubertally defeminized males (*ATD/55/T*). This indicates that a masculinization has followed the defeminization which had occurred between day 35 and 55. It is apparent however, that testosterone is sufficient to masculinize the adult velocity of 2α-hydroxylase in the absence of either neonatal or peripubertal defeminization. When *ATD/21* and *ATD/21/T* are compared, testosterone is responsible for a significant ($p<0.05$) increase in the velocity of 2α-hydroxylase to reach intact male levels. In this case, 2α-hydroxylation specific activity increased 4.5-fold, from 0.32 (± 0.125) to 1.44 (± 0.067) ($p<0.05$).

Because it is possible for testosterone fully masculinize the velocity of 2α-hydroxylase in the absence of neonatal or peripubertal defeminization, it is difficult

to determine whether any further imprinting of testosterone responsiveness occurs due to the peripubertal presence of testicular secretions. In other words, this response to testosterone in the absence of any form of defeminization may mask any peripubertal defeminization that may occur.

The fact that the *ATD/intact* (1.89 ± 0.015) was not significantly different from the *intact male* ($p<0.05$), indicates that testicular secretions are sufficient to cause a full masculinization of 2α-hydroxylase. The observation that the *ATD/55/T* (1.96 ± 0.165) and the *ATD/21/T* (1.44 ± 0.067) were not significantly different ($p<0.05$) than the *intact male* (1.66 ± 0.170) regarding the specific activity of 2α-hydroxylase again indicates that testosterone itself is sufficient to cause full masculinization.

4.3.4. P-450 2C11: 16α-hydroxylase (Table 4.6.D.):

Defeminization of a basal specific activity: A permanent peripubertal defeminization process can be seen as a significant ($p<0.05$) 2.7-fold increase in basal 16α-hydroxylase specific activity imprinted from day 35 (0.30 ± 0.103) to 55 (0.81 ± 0.021) in non-neonatally-defeminized males. No significant increase was seen in non-defeminized males whose testes remained *in situ* from day 21 (0.16 ± 0.040) to day 35 (0.30 ± 0.103). Interestingly, like that of 2α-hydroxylase, the specific activity of 16α-hydroxylase drops significantly ($p<0.05$) in non-neonatally-defeminized males whose testes remain *in situ* from day 55 (0.81 ± 0.021) to day 70 (0.54 ± 0.047).

Response to testosterone in adulthood: Non-neonatally-defeminized males castrated on day 55 showed a significant increase ($p<0.10$) in 16α-hydroxylase specific activity when exposed to testosterone in adulthood (from 0.81 ± 0.021 to 1.15 ± 0.174). This suggests that a masculinization followed the peripubertal defeminization, or as in the case of 2α-hydroxylase, we are just seeing the unimprinted ability of this enzyme to respond to testosterone in adulthood.

The fact that the *ATD/intact* (1.08 ± 0.105) was not significantly different from the *intact male* (0.98 ± 0.149), indicates that testicular secretions are sufficient to cause a full masculinization of 16α-hydroxylase. The observation that the *ATD/55/T* (1.15 ± 0.174) and the *ATD/21/T* (0.88 ± 0.032) were not significantly different ($p<0.05$) than the *intact male* (0.98 ± 0.149) indicates that testosterone itself is sufficient to cause this full masculinization.

4.4. Section four

"What length of time do the testes have to remain in place for this peripubertal event to occur?"

Once we ensured that neonatal defeminization had been blocked, we then attempted to investigate the effects of intact testes on the defeminization of basal velocities of the specific P-450s during specific time frames in the peripubertal window (*i.e.* days 21-70).

4.4.1. P-450 3A2: Ethylmorphine demethylase and 6β-hydroxylase (Tables 4.6.A. and 4.6.B.): The specific activities of both EMDM and 6β-hydroxylase were determined in animals who had neonatal defeminization blocked by neonatal ATD treatment (day 0-21), and had been castrated at age 21 or 35 days. No significant differences in EMDM specific activity were seen between the animals castrated on day 21 (1.7 ± 0.19) and day 35 (2.1 ± 0.21). Similarly, a slight ($p=0.082$) but not significant increase was seen in 6β-hydroxylase specific activity in animals castrated on day 35 (0.11 ± 0.042) as compared to those castrated on day 21 (0.002 ± n.d.). This indicates that no defeminization/masculinization had occurred due to the presence of intact testes between days 21 and 35.

A significant ($p<0.05$) 1.9-fold increase was noted in EMDM specific activity between animals castrated at day 35 (2.1 ± 0.21) and those castrated at day 55 (4.0 ± 0.14), indicating that a defeminization process had occurred. To determine whether or not this process had been completed by day 55 or required the testes to remain *in situ* until day 70 (adulthood), the *ATD/55* was compared with the *ATD/70*. The specific activity of EMDM did not change between day 55 (4.0 ± 0.14) and day 70 (3.9 ± 0.50), indicating that the defeminization process was completed by day 55.

A significant ($p<0.05$) 2.5-fold increase was noted in 6β-hydroxylase specific activity between animals castrated at day 35 (0.11 ± 0.042) and those castrated at day 55 (0.27 ± 0.022), indicating that a defeminization process had occurred. The presence of the testes from day 55 to day 70 resulted in a *decrease* in 6β-hydroxylase activity, from 0.27 (± 0.022) to 0.07 (± 0.055).

4.4.2. P-450 2C11: 16α-hydroxylase, 2α-hydroxylase (Tables 4.6.C. and 4.6.D.):

No significant differences were seen in the specific activities of 16α-hydroxylase or 2α-hydroxylase between animals castrated on day 21 and day 35. 16α-hydroxylase activity increased slightly but not significantly, from 0.16 (± 0.040) to 0.30 (± 0.103). The same occurred with 2α-hydroxylase activity, which increased slightly but not significantly from 0.32 (± 0.125) to 0.55 (± 0.192). This indicated that no apparent defeminization had occurred in this 14-day time-frame. Significant increases ($p<0.05$) in specific activities were seen in both 16α- and 2α-hydroxylations in animals castrated on day 55 as compared with day 35. The activity of 16α-hydroxylase increased 2.7-fold, from 0.30 (± 0.103) to 0.81 (± 0.021). Similarly, the activity of 2α-hydroxylase increased 2.4-fold, from 0.55 (± 0.192) to 1.32 (± 0.057). There was no significant increase in 2α-hydroxylase specific activity between day 55 (1.32 ± 0.057) and 70 (0.96 ± 0.092), as well as with 16α-hydroxylation (0.81 ± 0.021 on day 55 and 0.54 ± 0.047 on day 70). Instead, there appears to be a significant ($p<0.05$) *reduction* in 16α- (28%) and 2α- (33%) hydroxylation specific activities during this time frame.

4.5. The effect of sex hormone manipulation on other P-450-related isozymes and cofactors

4.5.1. Total hepatic P-450 content (Table 4.6.E.): The cytochrome P-450 content showed a slight but significant difference between males and females, with the *intact male* (1.03 ± 0.089) being greater than the *intact female* (0.73 ± 0.070). The cytochrome P-450 content was not responsible for any differences seen between groups in this experiment, as all groups (except for the *intact female*) were not significantly different from each other or the *intact male*. The small difference between the intact female and male could not have accounted for such large sex differences seen in 3A2 or 2C11.

4.5.2. NADPH Cytochrome *c* reductase (Table 4.6.F.): There was a small but significant ($p<0.05$) difference between the *intact female* (44.1 ± 2.42) and the *intact male* (53.2 ± 2.46), the female being less. The non-defeminized *adult castrate* (41.3 ± 3.42) was not significantly different than that of the *intact female* (44.1 ± 2.42) and also significantly less than the *intact male* (53.2 ± 2.46) suggesting that a lack of testosterone in the adult causes a slight reduction in reductase activity. The *ATD/21* (65.7 ± 3.85) and the *ATD/21/T* (59.8 ± 5.56) were also significantly greater than the *intact female* (44.1 ± 2.42) and the *adult castrate* (41.3 ± 3.42). Unlike in the defeminized male, this suggests that the absence of testosterone in the *non*-defeminized male does *not* cause a reduction in reductase activity. Whether or not

defeminization plays a role or not in ultimate adulthood reductase activity cannot be determined from these results.

4.5.3. <u>P-450 2E1: Aniline hydroxylase (Table 4.6.G.)</u>: Our data indicate that the activity of aniline hydroxylase (P-450 2E1) is sexually differentiated, with female activity (0.23 ± 0.052) being statistically less than ($p<0.05$) male activity (0.91 ± 0.077). This was not suprising, as some literature indicates a clear sex difference (Yamazoe *et al.*, 1989). This sex difference is also in agreement with Kato *et al.* (1968), but not in agreement with Virgo (1985), Schenkman *et al.* (1967), Finnen and Hassall (1980), and Shimada *et al.* (1987), who did not find any sex difference in aniline hydroxylase activity in the adult male and female rat. Neonatal castration has been found to be ineffectual in changing aniline hydroxylase activity (Schenkman *et al.*, 1967; Shimada *et al.*, 1987), and castration at any age thereafter also had no effect (Finnen and Hassall, 1980).

Our data, on the other hand, indicated that adult castration decreased aniline hydroxylase activity, by 33%, from (0.91 ± 0.08 to 0.57 ± 0.06), when intact males were castrated in adulthood (day 70), suggesting that the presence of testicular factors may directly/indirectly regulate this isozyme. It is probably not strictly testosterone that regulates aniline hydroxylase activity, as the exposure of the ATD-treated males to testosterone in adulthood did not significantly *increase* the activity of this enzyme (*ATD/21 = ATD/21/T*; *ATD/55 = ATD/55/T*; *ATD/70 = ATD/intact*). Since the defeminized empty/21 was not statistically different than the non-defeminized

ATD/21 (0.67 ± 0.180 *vs.* 0.89 ± 0.071), this indicates that neonatal ATD administration did not affect basal aniline hydroxylase activity. In addition, the activities of all ATD-treated males regardless of castration/treatment (except *ATD/35* and *adult castrate*), were not statistically different from that of the intact male, also indicating that the activity of P-450 2E1 is not imprinted and/or regulated in the same manner as the sex-specific P-450 3A2 and 2C11 enzymes.

Virgo (1991) found that testosterone propionate administration to the non-defeminized male, from day 35-71, did not affect aniline hydroxylase activity, which is in accordance with our data (*ATD/intact = intact male*). We also found that the presence of the testes (or testosterone) in the non-defeminized male (*ATD/21/T, ATD/55/T, ATD/intact*), did *not* significantly increase aniline hydroxylase specific activity over that of the castrated males (*ATD/21, ATD/55, ATD/70*).

4.5.4. <u>P-450 2A1: 7α-hydroxylase (Table 4.6.H.):</u> There was an expected (Kato *et al.*, 1986; Sonderfan *et al.*, 1987) significant difference between *intact males* (0.26 ± 0.073) and *intact females* (1.06 ± 0.169) in the specific activity of 7α-reductase (females > males).

<u>Adult masculinization:</u> Castration of adult intact male, resulted in a significant 3.1-fold increase (feminization) in 7α-hydroxylation specific activity as compared with the *intact male* group (0.81 ± 0.011 vs. 0.26 ± 0.073) ($p<0.05$). The final specific activity was not statistically different than that of the *intact female* group (1.06 ± 0.169), or the *empty/21* group (0.56 ± 0.069). This indicates that the

presence of testicular secretions accounts either directly, or indirectly, for the difference in 7α-hydroxylase activity, between the neonatally defeminized male (*adult castrate, empty/21*), and the *intact female*. This is supported by Waxman *et al.* (1989), who demonstrated that adult castration increased 2A1 levels.

The non-defeminized male: Waxman *et al.* (1989), and Shimada *et al.* (1987), reported a significant feminization (increase in activity) of 2A1 and 7α-hydroxylase levels/activity (respectively), in the neonatally castrated (non-defeminized) male. Adult testosterone administration to the non-defeminized male, has been shown to suppress (masculinize) 7α-hydroxylation (Shimada *et al.*, 1987), which indicates that testosterone is effecting the suppression. Therefore, neonatal castration prevents the normal peripubertal suppression (Sonderfan *et al.*, 1987) of 7α-hydroxylase.

Interestingly, our data showed that neonatal ATD administration may have blocked a *feminization* of 7α-hydroxylase. This is demonstrated in the *empty/21* vs. the *ATD/21*. We found that the male who did *not* receive ATD (and thus was exposed to estrogens), was significantly *feminized* (0.56 ± 0.069), whereas the ATD-treated male (who was not exposed to estrogens), was significantly *masculinized* (0.28 ± 0.034). This may provide evidence that estrogens serve to feminize 7α-hydroxylase activity in adulthood. Why we demonstrated male 7α-hydroxylase activity when defeminization was blocked, and Shimada *et al.* (1987) reported a feminization of 7α-hydroxylase activity in the neonatal castrate, is not known.

Our data showed that in the non-defeminized male, castration at 21 days (0.28 ± 0.034) vs. 35 days (0.68 ± 0.172), did *not* significantly increase (feminize) the 7α-hydroxylase specific activity. Castration of the ATD-treated males on day 55 (0.62 ± 0.034) did not significantly change the specific activity from that of the day 35 castrates ($p<0.05$), however, it resulted in significantly greater (more feminine) activity *vs.* the ATD/21 ($p<0.05$). The large standard error in the ATD/35 made it difficult to fully characterize this group. The activity of the ATD/70, was not statistically different from the *intact male* group (0.26 ± 0.073) ($p<0.05$). This indicates that, in the non-defeminized male, *basal* 7α-hydroxylase activities are masculine, with the exception of the day 55 castrate, who was neither feminine nor masculine.

Exposure of the non-defeminized males to testosterone in adulthood, did result in significant decreases ($p<0.05$) (masculinization) in the specific activity of 7α-hydroxylase, in the day 55 castrates (*ATD/55 > ATD/55/T*), and the day 70 castrates (*ATD/70 > ATD/intact*). Castration of ATD-treated males on day 21, however, and subsequent exposure to testosterone in adulthood, did not have any significant effect on 7α-hydroxylase activity (*ATD/21 = ATD/21/T*). The lack of statistical difference between these two groups, is probably due to the large standard error in the *empty/21* group. These data indicate that, in the non-defeminized male, testosterone in adulthood serves to suppress 7α-hydroxylase activity.

The presence of the testes throughout puberty in the non-defeminized male, resulted in masculine activities of 7α-hydroxylase (*ATD/intact*; our data). This

corresponds with Waxman *et al.* (1989), who found that pubertal testosterone administration to the non-defeminized male, decreased 7α-hydroxylase activity almost to that of the intact male (Waxman *et al.*, 1989). Shimada *et al.* (1987) also found that peripubertal testosterone administration (day 56-70) to the non-defeminized male, resulted in masculine 7α-hydroxylase activities.

TABLE 4.6.A. Hepatic ethylmorphine demethylase specific activity in rats of varying endocrine status. See **Table 2.2.B.** for individual treatment descriptions.

GROUP *(n = 4-7)*		**EMDM specific activity** *(nmol/min/mg microsomal protein)*
intact female	**a**	$1.4 \pm 0.11^{d,e,f,g,h,i,j,k}$
empty/21	**b**	$2.9 \pm 1.14^{g*,i,k}$
ATD/21	**c**	$1.7 \pm 0.19^{d,f,g,h,i,k}$
ATD/21/T	**d**	$4.7 \pm 0.46^{a,c,e,i,j,k}$
ATD/35	**e**	$2.1 \pm 0.21^{a,d,f,g,h,i,k}$
ATD/55	**f**	$4.0 \pm 0.14^{a,c,e,g*,i,j,k}$
ATD/55/T	**g**	$5.7 \pm 0.79^{a,b*,c,e,f*,h*,i,j}$
ATD/70	**h**	$3.9 \pm 0.50^{a,c,e,g*,i,j,k}$
ATD/intact	**i**	$8.2 \pm 0.59^{a,b,c,d,e,f,g,h,j}$
adult castrate	**j**	$2.2 \pm 0.15^{a,d,f,g,h,i,k}$
intact male	**k**	$7.2 \pm 0.47^{a,b,c,d,e,f,h,j}$

All data are the mean ± S.E.M..

* $p<0.10$

Superscripts corresponding with treatment letter designations represent a statistically significant difference in specific activity between compared groups, using the student *t*-test. All $p<0.05$ unless otherwise noted (*).

TABLE 4.6.B. Hepatic 6β-hydroxylase specific activity in rats of varying endocrine status. See **Table 2.2.B.** for individual treatment descriptions.

GROUP *(n = 2-4)*		**6β-hydroxylase specific activity** *(nmol/min/mg microsomal protein)*
intact female	**a**	$0.06 \pm 0.035^{d,f,g,i*,k}$
empty/21	**b**	0.18 ± 0.086
ATD/21	**c**	$0.002 \pm$ n.d. ^{d,e*,f,g,i,j,k}
ATD/21/T	**d**	$0.34 \pm 0.030^{a,c,e,h,j}$
ATD/35	**e**	$0.11 \pm 0.042^{c*,d,f,g,k*}$
ATD/55	**f**	$0.27 \pm 0.022^{a,c,e,h,j}$
ATD/55/T	**g**	$0.31 \pm 0.046^{a,c,e,h,j*}$
ATD/70	**h**	$0.07 \pm 0.055^{d,f,g,k*}$
ATD/intact	**i**	$0.26 \pm 0.143^{a*,c,f}$
adult castrate	**j**	$0.13 \pm 0.010^{c,d,f,g*}$
intact male	**k**	$0.28 \pm 0.062^{a,c,e*,h*}$

Data are the mean ± S.E.M..

* $p<0.10$

Superscripts corresponding with treatment letter designations represent a statistically significant difference in specific activity between compared groups, using the student *t*-test. All $p<0.05$ unless otherwise noted.

TABLE 4.6.C. Hepatic 2α-hydroxylase specific activity in rats of varying endocrine status. See **Table 2.2.B.** for individual treatment descriptions.

GROUP *(n = 2-4)*		**2α-hydroxylase specific activity** *(nmol/min/mg microsomal protein)*
intact female	**a**	$0.24 \pm 0.044^{d,f,g,h,i,j,k}$
empty/21	**b**	1.08 ± 0.468
ATD/21	**c**	$0.32 \pm 0.125^{d,f,g,h,i,j,k}$
ATD/21/T	**d**	$1.44 \pm 0.067^{a,c,e,g,h,i,j}$
ATD/35	**e**	$0.55 \pm 0.192^{d,f,g,i,k}$
ATD/55	**f**	$1.32 \pm 0.057^{a,c,e,g,h,i,j,k*}$
ATD/55/T	**g**	$1.96 \pm 0.165^{a,c,d,e,f,h,j}$
ATD/70	**h**	$0.96 \pm 0.092^{a,c,d,f,g,i,j,k}$
ATD/intact	**i**	$1.89 \pm 0.015^{a,c,d,e,f,h}$
adult castrate	**j**	$1.00 \pm 0.028^{a,c,d,f,g,h,k*}$
intact male	**k**	$1.66 \pm 0.170^{a,c,e,f*,h,j*}$

All data are the mean ± S.E.M..

* $p<0.10$

Superscripts corresponding with group letter designations represent statistically significant differences in specific activity between compared groups, using the student *t*-test. All $p<0.05$ unless otherwise noted (*).

TABLE 4.6.D. Hepatic 16α-hydroxylase specific activity in rats of varying endocrine status. See **Table 2.2.B.** for individual treatment descriptions.

GROUP *(n = 2-4)*		**16α-hydroxylase specific activity** *(nmol/min/mg microsomal protein)*
intact female	**a**	$0.15 \pm 0.044^{d,f,g,h,i,j,k}$
empty/21	**b**	0.68 0.315
ATD/21	**c**	$0.16 \pm 0.040^{d,f,g,h,i,j,k}$
ATD/21/T	**d**	$0.88 \pm 0.032^{a,c,e,h,i*,j}$
ATD/35	**e**	$0.30 \pm 0.103^{d,f,g,i,k}$
ATD/55	**f**	$0.81 \pm 0.021^{a,c,e,g*,h,j}$
ATD/55/T	**g**	$1.15 \pm 0.174^{a,c,e,f*,h,j*}$
ATD/70	**h**	$0.54 \pm 0.047^{a,c,d,f,g,i,k}$
ATD/intact	**i**	$1.08 \pm 0.105^{a,c,d*,e,j,h}$
adult castrate	**j**	$0.48 \pm 0.039^{a,c,d,f,g*,i,k*}$
intact male	**k**	$0.98 \pm 0.149^{a,c,d,e,h,j*}$

All data are the mean ± S.E.M..

* $p<0.10$

Superscripts corresponding with treatment letter designations represent a statistically significant difference in specific activity between compared groups, using the student *t*-test. All $p<0.05$ unless otherwise noted (*).

TABLE 4.6.E. Hepatic cytochrome P-450 content in rats of varying endocrine status. See **Table 2.2.B.** for individual treatment descriptions.

GROUP *(n = 4-7)*		**Cytochrome P-450 content** *(nmol/mg microsomal protein)*
intact female	**a**	$0.73 \pm 0.070^{c,d,i,j,k}$
empty/21	**b**	0.89 ± 0.135
ATD/21	**c**	$1.05 \pm 0.112^{a,f,g*}$
ATD/21/T	**d**	$1.06 \pm 0.059^{a,e,f,g,h}$
ATD/35	**e**	$0.86 \pm 0.039^{d,f*,i,j*}$
ATD/55	**f**	$0.72 \pm 0.053^{c,d,e*,h*,i,j,k}$
ATD/55/T	**g**	$0.76 \pm 0.073^{c*,d,i,j,k*}$
ATD/70	**h**	$0.83 \pm 0.037^{d,f*,i,j,k*}$
ATD/intact	**i**	$1.01 \pm 0.053^{a,e,f,g,h}$
adult castrate	**j**	$1.05 \pm 0.020^{a,e,f,g,h}$
intact male	**k**	$1.03 \pm 0.092^{a,f,g*,h*}$

Data are the mean ± S.E.M..

* $p<0.10$

Superscripts corresponding with treatment letter designations represent a statistically significant difference in P-450 content between compared groups, using the student *t*-test. All $p<0.05$ unless otherwise noted (*).

TABLE 4.6.F. Hepatic NADPH cytochrome *c* reductase specific activity in rats of varying endocrine status. See **Table 2.2.B.** for individual treatment descriptions.

GROUP *(n = 4-7)*		**Cytochrome *c* reductase specific activity** *(nmol/min/mg microsomal protein)*
intact female	**a**	44.1 ± 2.42 ^{c,d,e*,f*,g,k}
empty/21	**b**	58.8 ± 8.69
ATD/21	**c**	65.7 ± 3.85 ^{a,e*,f,g*,h,j,k}
ATD/21/T	**d**	59.8 ± 5.56 ^{a,j}
ATD/35	**e**	53.9 ± 4.07 ^{a*,c*,j*}
ATD/55	**f**	52.3 ± 3.10 ^{a*,c,j}
ATD/55/T	**g**	54.8 ± 3.60 ^{a,c*,j}
ATD/70	**h**	51.6 ± 4.76 ^{c}
ATD/intact	**i**	63.4 ± 12.92
adult castrate	**j**	41.3 ± 3.42 ^{c,d,e*,f,g,k}
intact male	**k**	53.2 ± 2.46 ^{a,c,j}

Data are the mean ± S.E.M..

* $p<0.10$

Superscripts corresponding with treatment letter designations represent a statistically significant difference in specific activity between compared groups, using the student *t*-test. All $p<0.05$ unless otherwise noted (*).

TABLE 4.6.G. Hepatic aniline hydroxylase specific activity in rats of varying endocrine status. See **Table 2.2.B.** for individual treatment descriptions.

GROUP *(n = 4-7)*		**Aniline hydroxylase specific activity** *(nmol/min/mg microsomal protein)*
intact female	**a**	$0.23 \pm 0.052^{b,c,d,e,f,g,h,i,j,k}$
empty/21	**b**	0.67 ± 0.180^{a}
ATD/21	**c**	$0.89 \pm 0.071^{a,e,g,j}$
ATD/21/T	**d**	$0.87 \pm 0.094^{a,e,g*,j}$
ATD/35	**e**	$0.54 \pm 0.056^{a,c,d,f,i,k}$
ATD/55	**f**	$0.76 \pm 0.057^{a,e,j*}$
ATD/55/T	**g**	$0.68 \pm 0.054^{a,c,d*,j*,k}$
ATD/70	**h**	0.71 ± 0.100^{a}
ATD/intact	**i**	$1.01 \pm 0.158^{a,e,g*,j*}$
adult castrate	**j**	$0.57 \pm 0.060^{a,c,d,f*,j*,k}$
intact male	**k**	$0.91 \pm 0.077^{a,e,g,j}$

Data are the mean ± S.E.M..

* $p<0.10$

Superscripts corresponding with treatment letter designations represent a statistically significant difference in specific activity between compared groups, using the student *t*-test. All $p<0.05$ unless otherwise noted (*).

TABLE 4.6.H. Hepatic 7α-hydroxylase specific activity in rats of varying endocrine status. See **Table 2.2.B.** for individual treatment descriptions.

GROUP *(n = 4-7)*		**7α-hydroxylase specific activity** *(nmol/min/mg microsomal protein)*
intact female	**a**	1.06 ± 0.169 [b,c,d,f,g,h,i,k]
empty/21	**b**	0.56 ± 0.069 [a,c,i,k]
ATD/21	**c**	0.28 ± 0.064 [a,b,f,h,j]
ATD/21/T	**d**	0.47 ± 0.095 [a]
ATD/35	**e**	0.68 ± 0.172
ATD/55	**f**	0.62 ± 0.034 [a,c,g,h,i,j,k]
ATD/55/T	**g**	0.36 ± 0.062 [a,f,j]
ATD/70	**h**	0.44 ± 0.046 [a,c,f,i,j]
ATD/intact	**i**	0.19 ± 0.041 [a,b,f,h,j]
adult castrate	**j**	0.81 ± 0.011 [c,f,g,h,i,k]
intact male	**k**	0.26 ± 0.073 [a,b,f,j]

Data are the mean ± S.E.M..
Superscripts corresponding with treatment letter designations represent a statistically significant difference in specific activity between compared groups, using the student *t*-test. All $p<0.05$.

5.0. DISCUSSION

5.1. Our characterization of sex-specific P-450s 3A2 and 2C11 *(sex differences, neonatal defeminization, and masculinization)*

5.1.1. Sex differences: Our results demonstrated the expected sex differences (males > females) in 2C11 and 3A2 specific activity. We report a (5-fold) difference between males and females in EMDM metabolism. This is consistent with data from Finnen and Hassall (1980), Shimada *et al.* (1987), and Virgo (1991). We also report a (4.7-fold) sex difference in 6β-hydroxylase activity, consistent with Waxman *et al.* (1985), Waxman *et al.* (1988), and Shimada *et al.* (1987). Our (6.7-fold) difference in 16α-hydroxylase activity is consistent with Shimada *et al.* (1987), Waxman *et al.* (1989), and Waxman *et al.* (1985), and our (6.9-fold) sex difference in 2α-hydroxylase activity, corresponds with both Shimada *et al.* (1987), and Waxman *et al.* (1988). Our 7α-hydroxylase activity sex differences (females express 4-fold greater activity), is consistent with data from Shimada *et al.* (1987).

Past literature does not demonstrate a conclusive sex difference in the adult specific activity of aniline hydroxylase (MacLeod *et al.*, 1972; Shimada *et al.*, 1987), however our data indicated a clear sex difference, males exhibiting a 4-fold higher enzyme activity. These results are in accordance with those from Yamazoe *et al.* (1989) who also reported a significant sex difference in 2E1 mRNA levels (males > females). We also demonstrated significant sex differences in total P-450 content

(females are 75% of male levels), confirmed by MacLeod *et al.* (1972), as well as total cytochrome *c* reductase activity (females are 83% of male levels), in accordance with Virgo (1985).

5.1.2. Neonatal defeminization: Neonatal defeminization can be characterized in animals of three different endocrine status': ***(a)*** the *prepubertal castrate*, ***(b)*** the *adult castrate*, and ***(c)*** the *neonatal castrate (with neonatal androgen treatment)*. We demonstrated neonatal defeminization in our *empty/21* (prepubertal castrate), and also in our *adult castrate*. Intact adult males are not an accurate representation of neonatal defeminization, as these males have been exposed to testosterone both peripubertally as well as in adulthood.

EMDM: We suggest that neonatal defeminization is represented as a significant 1.6-fold increase in specific activity, over that of the intact adult female. Statistical significance was only apparent in the male castrated in adulthood when compared with the female (the standard error was relatively large in our other group which represented neonatal defeminization, the *empty/21*). Our data is supported by those of a number of laboratories, as the following literature indicates that neonatal defeminization may be characterized as an increase in EMDM specific activity, over that of either the neonatally castrated male or the intact female. Castration after the "critical neonatal period" has been shown to result in either no increase (Chung *et al.*, 1975), or in 1.3- and 2.2-fold increases in EMDM specific activity (Finnen and Hassall, 1980; Virgo, 1991), over that of the neonatally castrated

male (non-defeminized). Exogenous testosterone administration to the neonatally castrated male resulted in the same variation, either a 2-fold increase (Shimada *et al.*, 1987; Virgo, 1991), or none at all (Chung *et al.*, 1975).

By definition, the intact female has been deemed the "standard", representing the absence of neonatal defeminization. Whether or not the female can be *unequivocally* used as a marker for the presence or absence of neonatal defeminization of EMDM, has not been conclusively determined, although our data indicate that it can (*ATD/21 = intact female*). Shimada *et al.* (1987) and Virgo (1991) demonstrated that the EMDM activity in the non-defeminized male, is *not* different from that of the intact female, whereas the non-defeminized male has also been shown to exhibit EMDM specific activities greater than that of the female (Finnen and Hassall, 1980; Chung *et al.*, 1975). The latter suggests that there may be other factors, aside from those arising from the testes, which may be involved in the neonatal defeminization process.

6β-hydroxylase: We demonstrate neonatal defeminization of 6β-hydroxylase as an increase in specific activity to a level slightly, but not significantly, greater than that of the female. A significant difference was noted between the defeminized and non-defeminized male, upon comparing the *adult castrate* with the *ATD/21*. When compared with the *ATD/21*, which also was not defeminized, the activity in the neonatally defeminized *adult castrate* was significantly greater.

We support the concept that neonatal defeminization of 6β-hydroxylase, is most likely shown as an increase in specific activity over that of the non-defeminized

male. In other laboratories, neonatal defeminization (either endogenous or exogenous) resulted in 6β-hydroxylase activity up to 13-fold greater than the non-defeminized male (Waxman *et al.*, 1985; Shimada *et al.*, 1987; Waxman *et al.*, 1988). As the non-defeminized male, the *ATD/21* in our case, demonstrated a specific activity not greater than that of the intact female (Shimada *et al.* 1987; our data), it is likely that the female can also be used as a representative marker for the absence of neonatal defeminization in the male.

16α-hydroxylase: We report neonatal defeminization of 16α-hydroxylase, as a significant increase in specific activity to levels 3-fold greater than the *intact female*. Other laboratories reported neonatal defeminization as an increase in specific activity to levels up to 5-fold greater than the non-defeminized male (Shimada *et al.*, 1987; Waxman *et al.*, 1989). It was also suggested that the specific activity of 16α-hydroxylase in the non-defeminized male is not different from the intact female (Shimada *et al.*, 1987), confirming our data, and also indicating that the female represents a non-defeminized male.

2α-hydroxylase: We report neonatal defeminization of 2α-hydroxylase as an increase in specific activity to levels 4-fold greater than those of the *intact female*. Other laboratories reported neonatal defeminization as an increase in specific activity to levels up to 8-fold greater than the non-defeminized male (Waxman *et al.*, 1985; Waxman *et al.*, 1988; Shimada *et al.*, 1987), and suggest that the non-defeminized male is equivalent to the intact female (Shimada *et al.*, 1987).

This confirms our data, and also indicates that the female can be used as a marker indicating the absence of neonatal defeminization.

5.1.3. Prevention of neonatal defeminization: If neonatal defeminization is characterized as an increase in basal enzyme activity to levels greater than that of the female, then we demonstrated that neonatal ATD administration is effective in preventing this defeminization process.

EMDM: Researchers indicate that neonatal castration effects an adult activity of EMDM either *equivalent* to that of the intact female (Shimada *et al.*, 1987; Virgo, 1991), or slightly greater than that of the intact female (Chung *et al.*, 1975; Finnen and Hassall, 1980). Neonatal ATD treatment resulted in EMDM specific activity that was not different than that of the female, suggesting that neonatal defeminization had been prevented. Two additional lines of reasoning which indicate that defeminization had been blocked, are *(a)* adult testosterone administration to the *ATD/21* did not restore adult activity, as Chung *et al.* (1975) suggested that it should, in the *defeminized* male (also restoring K_m and V_{max} levels; Chung, 1977), and *(b)* neonatal ATD administration has been shown to prevent both the neonatal defeminization of sexual and behavioural characteristics (Morali *et al.*, 1977; Bakker *et al.*, 1995; Swaab *et al.*, 1995), and neonatal defeminization of EMDM (Reyes and Virgo, 1988).

6β-hydroxylase: Our data show that ATD administration prevented any increase in basal enzyme expression over that of the female. 6β-

hydroxylation in the non-defeminized male (also castrated on day 21), was significantly *less* than the neonatally defeminized *adult castrate*, also indicating that defeminization had been prevented.

16α-hydroxylase and 2α-hydroxylase: Neonatal defeminization of both reactions, was conclusively prevented by ATD administration. Defeminization was seen as an increase in 16α- and 2α-hydroxylase specific activities over those of the *intact female* and we demonstrated that neonatal ATD administration prevented such an increase, and thus prevented defeminization. These data were further supported by the observation that the specific activities exhibited by the *ATD/21* (non-defeminized, castrated on day 21), were significantly *less* than those of the neonatally defeminized *adult castrate*.

5.1.4. Neonatal ATD administration: We demonstrated the effectiveness of ATD delivery in Silastic™ tubing, consistent with data from Swaab *et al.* (1995), and Vreeburg *et al.* (1977).

From our data, we also conclude that the time-frame for administration of ATD (day 0 - day 21), was sufficient to prevent neonatal defeminization of these isozymes. As our animals were not castrated, the amount of time that the ATD capsule remained *in situ* was of importance, as Davis *et al.* (1995) suggested that the critical period for the *removal* of endogenous gonadal steroids on the resultant sexual differentiation of the SDN-POA, did not match that for the administration of *exogenous* gonadal steroids. We kept the ATD capsule *in situ* for 21 days, and

demonstrated that the 2C11 and 3A2 *empty/21* activity (castration at 21 days) equaled that of the *adult castrate* (castration at 70 days). Thus, it is reasonable to assume that the critical period for *removal of endogenous steroids* is enveloped in days 0-21, as keeping the testes intact for an additional 49 days did not result in any further increase in basal enzyme activity.

5.1.5. Masculinization in the neonatally defeminized male: We report that the *reversible* masculinization process, accounts for 31% of EMDM intact male activity, and 46% of 6β-hydroxylase activity [100 – (adult castrate ÷ intact male × 100)]. Adult castration has been shown to decrease EMDM specific activity by up to 54%, the effects being completely reversible upon exogenous testosterone administration (Kramer *et al.*, 1979).

We also report that the reversible masculinization process, accounts for 40% of 2α-hydroxylase, and 51% of 16α-hydroxylase activities. Adult castration has been shown to significantly decrease 2C11 mRNA (16α-hydroxylase) levels (Janeczko *et al.*, 1990), the effect being completely reversed upon exogenous testosterone administration (Janeczko *et al.*, 1990), suggesting that testosterone effects reversible masculinizatinon.

The mechanism by which testosterone produces this reversible response, may be mediated by the effects of sex hormones on the pattern of growth hormone secretion (**section 1.4.3.**). Sex hormones have been shown to directly regulate GH patterns, as both adult castration or estradiol treatment of the intact male, can

partially feminize the GH secretion pattern by increasing it's trough levels (Mode *et al.*, 1982; Carlsson *et al.*, 1987). The effects of castration can be reversed upon testosterone therapy (Mode *et al.*, 1982). Thus, the increase in trough levels produced by castration, could partially feminize the GH secretory pattern, and because low trough levels are necessary for 2C11 expression (Waxman *et al.*, 1991), 2C11 expression would subsequently decrease.

The change in 2C11 expression may be related to the dose (*i.e.* mean plasma concentration) of GH, as Wells *et al.* (1994) found that 2C11 expression (mRNA) in the male rat, was modified by GH in a dose-dependent manner. In other words, as the dose of GH *increased*, the amount of 2C11 mRNA *decreased*. It is possible, then, that sex hormone modification of the GH secretory profile (increasing baseline levels), may be correlated with the ultimate expression of 2C11 (*i.e.* very low trough levels cause full expression, whereas increasing trough levels cause decreasing 2C11 expression). This is supported by Waxman *et al.* (1988), who found that exogenous GH therapy at a *lower* dose (intermittently injected), was more effective than the *higher* dose, at reversing the hypophysectomy-induced increase and decrease, in 6β- and 2α-hydroxylase activity respectively.

A similar dose-dependence mechanism acting on EMDM, was found in the Kramer *et al.* (1978) study. They found that, in the castrated male, increasing the dosage of exogenous estradiol, caused a concomitant decrease in ethylmorphine metabolism. They also found that the increasing doses of estradiol decreased the activity of cytochrome *c* reductase. Estradiol administration, coupled with

continuous GH infusion, did not *further* decrease EMDM activity, suggesting that the two hormones act through the same mechanism to effect the decrease in activity. This indicates that estradiol is probably mediating its actions *via* modulation of the GH secretion pattern.

5.1.6. Adult responsiveness in the absence of neonatal defeminization: In the *absence* of neonatal defeminization (*ATD/21*), we report that all four reactions (two isozymes) are somewhat responsive to testosterone administration in adulthood (*ATD/21/T*). Virgo (1991) demonstrated that in the non-defeminized male (neonatal castrate), adult administration of testosterone had no effect (increase) on the velocity of EMDM. In other words, the ability of testosterone to increase the EMDM V_{max}, is *absent* in non-defeminized males. We demonstrated that in the non-defeminized male the specific activity of EMDM increased 2.8-fold, when administered testosterone in adulthood, but did not reach 100% adult male activities. The testosterone hydroxylases, on the other hand, responded *completely* to adult testosterone administration, reaching *full* male intact levels. In other words, the V_{max} of each system could be regulated either directly or indirectly (perhaps through modulation of the growth hormone pattern) by testosterone, in the absence of defeminization. We will subsequently refer to this unique response in the absence of defeminization, as **maleness**.

The observation that 2C11 and 3A2 responded similarly to testosterone administration (*i.e.* by an increase in activity), supports the concept that testosterone

modulates these isozymes through the same mechanism (perhaps through the GH pattern). On the other hand, the observation that the EMDM responded *incompletely* (not to 100% intact male activity) to testosterone treatment, may indicate that 3A2 is not *solely* regulated by sex-steroid modulation of the GH secretory pattern. Further research in this area would be of great interest, for we can only speculate, as we did not monitor growth hormone levels during our study.

Chung (1977) demonstrated that in the absence of neonatal defeminization the EMDM enzyme exhibits a slight (2-fold) increase in EMDM V_{max} when stimulated by testosterone in adulthood. Perhaps our EMDM demonstrated this characteristic. The 2α- and 16α-hydroxylases are dependent on the very low trough levels of the male GH secretory pattern for full expression in adulthood (Waxman *et al.*, 1991; Legraverend *et al.*, 1992b). Moreover, Jansson and Frohman (1987) demonstrated that in the absence of neonatal defeminization adult testosterone therapy could fully masculinize the GH secretory pattern (restoring low trough levels). Therefore, the ability of 2C11 (2α- and 16α-hydroxylases) activity to be completely masculinized by adult testosterone administered in the absence of neonatal defeminization, could possibly be due to the complete restoration of the GH secretory profile.

5.1.7. <u>Summary</u>: Defeminized P-450 enzyme characteristics can be characterized as an increase in basal enzyme activity to values greater than those of the non-defeminized male (or intact female). Our results indicate that neonatal defeminization of all four reactions (two isozymes), is characterized as a permanent

increase in basal enzyme activity, to levels greater than either the non-defeminized male, or the intact female. Neonatal ATD administration was effective in preventing this characteristic defeminization of 2C11 and 3A2. Defeminization may or may not be associated with a responsiveness (increase in activity) to adult testosterone administration (*i.e.* masculinization). Our results suggest that neonatal defeminization is *not* a necessary prerequisite for adult responsiveness (*i.e.* the ability of testosterone to increase the specific activity) of EMDM, nor 6β-, 2α-, and 16α-hydroxylases, as all enzymes were responsive to testosterone in adulthood, in the absence of defeminization (referred to as *maleness*). This suggests that androgen responsiveness is either ***(a)*** not neonatally imprinted *by estrogens* (*i.e.* may be imprinted by other testicular secretions which were not blocked by ATD), or ***(b)*** not a neonatally imprinted characteristic of 3A2 or 2C11. This indicates that not only are neonatal defeminization and adult masculinization not directly related, but they are also not imprinted/regulated by the same mechanism.

Adult testosterone treatment in the non-defeminized male, was effective at restoring full intact male testosterone hydroxylase velocities, however this hormone was not sufficient to fully restore adult male activities of EMDM. These data suggest that another hormone (possibly estrogens) may effect the defeminization of an ability of EMDM to reach intact male levels following testosterone administration (as blocking neonatal estrogen production prevented the testosterone-stimulated velocity). It is likely that estrogens are responsible for defeminizing the V_{max} of EMDM, as Reyes and Virgo (1988) demonstrated the ability of ***(a)*** an estrogen

receptor blocker, and/or ***(b)*** an aromatase inhibitor (ATD), to prevent the neonatal defeminization of a fully masculine EMDM V_{max}. They also demonstrated that blocking the androgen receptor, during the neonatal critical period, did not prevent the imprinting of a male V_{max}. This corresponds with the theory of estrogen-mediated defeminization of the rat brain (**section 1.3.**).

5.2. Peripubertal imprinting in the absence of neonatal defeminization

Previous workers have concluded that neonatal defeminization is a ***necessary prerequisite*** for the adult masculinization (*i.e.* responsiveness to testosterone) of sex-specific P-450s, (Chung *et al.*, 1975, Virgo, 1991). Our results suggest that this is not a correct conclusion. We demonstrated that, in the absence of neonatal defeminization, 3A2 and 2C11 were responsive to testosterone in adulthood (*maleness*). We also demonstrated that maximum intact male activities were obtainable following testosterone administration in adulthood, for the 6β-, 2α-, and 16α-hydroxylase enzymes, but not for the EMDM. The differences seen in EMDM and 6β-hydroxylase responses to hormone manipulation may be due to the additional isozymes responsible for the 6β-hydroxylation of testosterone (**section 1.1.**). These isozymes may not be responsive to hormonal manipulation (*i.e.* not defeminized and/or regulated in the same manner as 3A2), and neonatal ATD administration may not have altered their adult activities.

More importantly however, in the absence of neonatal defeminization, we demonstrated that peripubertal testicular secretions are ***(a)*** sufficient to *completely* defeminize and masculinize (*i.e.* to intact male activities) 2C11 and 3A2, and ***(b)*** effective at imprinting (*i.e.* defeminizing) basal EMDM, 6β-hydroxylase, 2α-hydroxylase, and 16α-hydroxylase activities, to levels significantly greater than that of the female. Our data confirm, and add to, those from Dannan *et al.* (1986), Shimada *et al.* (1987), Waxman *et al.* (1989), Virgo (1991), and Bandiera and Dworschak (1992), who found that peripubertal administration of testosterone to the non-defeminized male, resulted in a complete "masculinization" of EMDM, 6β-, 2α-, and 16α-hydroxylase specific activities.

5.2.1. <u>Our characterization of peripubertal "masculinization"</u>: Certain methods carried out in this study, were designed to assist in characterization of this peripubertal masculinization phenomenon. In other words, to define specific components of this event, and possibly give insight into the mechanisms involved. We describe two major characteristics of peripubertally "masculinized" enzymes, being a *reversible* component (masculinization), and a *permanent* component (peripubertal defeminization).

<u>Permanent component</u>: Data obtained in this study indicated that our leaving the testes intact, revealed a permanent component of the peripubertal "masculinization" phenomenon. This is apparent as a permanent increase in basal enzyme activity, to activities significantly greater than those of the non-defeminized

(*ATD/21*) male and/or *intact female*. We will henceforth refer to this permanent increase in basal specific activity, as **peripubertal defeminization**. We demonstrated peripubertal defeminization of EMDM activity, and also of 6β-, 2α-, and 16α-hydroxylase activity (*i.e.* 3A2 and 2C11).

Virgo *et al.* (1991) demonstrated that removal of the peripubertal testosterone treatment in the non-defeminized male, completely reversed the effects of the peripubertally (35-71) administered testosterone, on EMDM metabolism (*i.e.* lack of defeminization, only masculinization). We demonstrated that removal of the testes on either day 55 or day 70, did *not* completely reverse the peripubertal masculinization of EMDM metabolism, and suggest that other testicular factors present in the *intact animal* (*vs.* exogenously administered testosterone), may be responsible for the peripubertal defeminization of basal enzyme activity. This may indicate that there was either ***(a)*** a significant difference in peripubertal defeminization of the *intact* male *vs.* the *exogenous* peripubertal defeminization of the *castrate*, or ***(b)*** our method of blocking neonatal defeminization (ATD) somehow allowed (*i.e.* was a necessary prerequisite for) peripubertal defeminization to occur.

We also demonstrated a permanent increase (defeminization) of basal 2α-, 16α-, and 6β-hydroxylase activities, if the testes were removed on day 55, but interestingly demonstrated a *reversal* of the peripubertal defeminization in males castrated on day 70. It is interesting that only the testosterone hydroxylases demonstrated this increase in basal enzyme activity, when castration occurred on day 55. The data from our study which indicated that castration of the non-defeminized

male on day 55, also resulted in a significant increase (feminization) in *7α-hydroxylase* activity (greater than that of the day 70 castrate), introduces the concept that some factor may be directly regulating P-450 enzyme levels at this particular time, either in addition to, or instead of the "normal" (*i.e.* GH-mediated) regulatory mechanisms. This suggests that ***(a)*** this peripubertal imprinting process may be a dynamic process that changes in relation to circulating testicular hormone at any given time (and is reflected in correlating basal enzyme activities), ***(b)*** some other mechanism engaged by the testicular secretions from day 55-70, essentially overrides, or reverses, this peripubertal defeminization process, or ***(c)*** this increase at day 55 is a hormonal "overshoot" phenomenon, characteristic of negative feedback systems.

Reversible component: In the ATD-treated and pubertally defeminized animals, we found that the degree of responsiveness of EMDM, and the 2α- and 16α-hydroxylases, to testosterone in adulthood, appeared to remain constant regardless of the age at castration. As noted earlier (**section 5.1.6.**), because our non-neonatally defeminized animal responded to testosterone in adulthood (*ATD/21/T*), it is difficult to determine whether this responsiveness was triggered by the peripubertal mechanism, or whether the response simply reflects a non-defeminized characteristic of these enzymes (*i.e.* pharmacological levels of testosterone in the non-defeminized male resulted in "maleness"). Our results indicate that exogenous testosterone administration cannot distinguish maleness (defeminization-*independent*) from masculinization (defeminization-*dependent*), as the *ATD/21/T* (non-defeminized plus maless) was equivalent to the *ATD/55/T* (peripubertally defeminized plus

masculinized). It appears however, that masculinization is greater (*i.e.* results in a higher specific activity) than maleness, as the EMDM specific activity of the intact male (defeminized and masculinized), was significantly larger than in the non-defeminized but testosterone-treated male (*ATD/21 T*).

The indication that the same pharmacological dose of testosterone effected the same magnitude of increase in specific activity (*ATD/21/T* vs. *ATD/55/T*) in the 2α-, and 16α-hydroxylases, may be indicative of the effects of testosterone on the GH pattern, and subsequent modulation of 2C11 activity. For example, castration of these animals (on either day 21 or 55) may have increased GH trough levels (Mode *et al.*, 1982), and subsequently decreased the activity of these isozymes. Testosterone therapy possibly restored the low GH trough levels (Mode *et al.*, 1982), and in so doing, may have effected an increase in 2C11 activity (Waxman *et al.*, 1991). It would have been interesting to see if there was a *lesser* increase in 2C11 activity, following a *smaller* dose of testosterone, as Wells *et al.* (1994) suggested a dose-dependent relationship between GH and 2C11.

5.2.2. <u>"Masculinization" *vs.* "Maleness"</u>: Up to this point, we have described three characteristics of sex-specific P-450s 2C11 and 3A2: *defeminization*, *masculinization*, and *maleness*. *Defeminization* is characterized as a permanent increase in basal enzyme activity (over that of the female), *masculinization* is characterized as a response (increase) to testosterone (dependent on defeminization), and *maleness* refers to a response (increase) to testosterone in the absence of

defeminization. Our data could not distinguish between maleness and masculinization of 2C11 and 6β-hydroxylase, as full male activities were reached in both the *absence* of neonatal defeminization as well as in the *presence* of peripubertal defeminization.

We did however, observe a possible difference between maleness and masculinization of EMDM. We found that the resultant activity following peripubertal masculinization (which by definition must follow peripubertal defeminization), was *greater* than the activity following testosterone-stimulated maleness (in the non-defeminized male). Based on this observation, we suggest that Virgo (1991) demonstrated an apparent masculinization of EMDM specific activity following peripubertal testosterone treatment in the non-defeminized male, as the non-defeminized male did *not* demonstrate maleness. This laboratory however, failed to demonstrate a peripubertal defeminization of EMDM, as the basal activity did not permanently increase following peripubertal testosterone treatment (but increased as a result of neonatal testosterone treatment). No evidence of peripubertal defeminization preceding masculinization has been demonstrated, with the exception of our data and those from Bandiera and Dworschak (1992). They found that peripubertal testosterone treatment (day 35-49) significantly increased 2C11 protein levels, over those of the exogenously neonatally defeminized male. The main reason why peripubertal defeminization has not been demonstrated in other laboratories, is most likely due to experimental design. Only our laboratory, Virgo (1991), and Bandiera and Dworschak (1992), removed the hormonal influence prior to animal

sacrifice. Thus, it is difficult to determine whether peripubertal defeminization occurred *prior* to masculinization in the other laboratories, as masculinization effectively "covers up" defeminization, and can only be demonstrated upon removal of testosterone influence. In addition, there may also exist a confounding effect due to "maleness". Although masculinization implies defeminization, and defeminization has not been *conclusively* demonstrated (with the exception of our laboratory), we will refer to their peripubertal phenomenon as **peripubertal masculinization**, for discussion purposes.

5.2.3. "Their" characterization of peripubertal masculinization: The complete peripubertal masculinization of 2C11 and 3A2, has been demonstrated in a number of laboratories, and has also been demonstrated in female as well as male rats (Dannan *et al.*, 1986; Shimada *et al.*, 1987; Waxman *et al.*, 1989; Virgo, 1991; Bandiera and Dworschak, 1992; Cadario *et al.*, 1992; Chang *et al.*, 1996; Chang and Bellward, 1996). For comparative purposes, the characterization of the peripubertal masculinization of these enzymes from other laboratories, will be related to the data from this study. Data from the research on the female rat (Cadario *et al.*, 1992; Chang and Bellward, 1996; Chang *et al.*, 1996) will be considered, for discussion purposes. As well, the information gathered from the peripubertal defeminization of ***(a)*** other male-specific enzymes (Chung *et al.*, 1975; Pak *et al.*, 1984; Pak *et al.*, 1985; Imamura *et al.*, 1994) will be considered.

Dannan *et al.* (1986) reported a complete masculinization of 16α-hydroxylase (and corresponding 2C11 levels), and 6β-hydroxylase activity (and corresponding 3A2 levels), in the non-defeminized male rat. The masculinization of the testosterone hydroxylases corresponded with the masculinization of their respective P-450 levels, which indicates that the peripubertal masculinization did not modify just one aspect (*i.e.* cofactor availability) of individual hydroxylase activity. Instead, it implies an increase in enzyme *level*.

Bandiera and Dworschak (1992), found that, as a percentage of total P-450, peripubertal (day 56-70) testosterone treatment of the non-defeminized male, effected a complete (relative to the exogenously defeminized/masculinized) masculinization of 2C11 levels. The administration of testosterone from day 35-49, to the exogenously neonatally defeminized male, and subsequent examination on day 70, revealed a permanent increase in 2C11 levels. Although the animal was defeminized, there was a significant increase over that of the defeminized male, following administration (and subsequent removal) of testosterone, during this time-frame. This observation may support our data indicating a permanent imprinting of an increase in basal 2C11 activity. The data indicating that the same peripubertal testosterone treatment of the neonatally castrated male *failed* to evoke a significant increase in basal enzyme levels, may support our claim that the presence of neonatal *androgens* (not *estrogens*), are a necessary prerequisite to effect peripubertal defeminization (*i.e.* our method of blocking estrogen, but not androgen, production allows for this peripubertal imprinting, whereas neonatal castration does not).

Shimada *et al.* (1987), found that 16α- and 2α-hydroxylase activities were almost completely restored in the non-neonatally-defeminized male, following testosterone administration from day 56-70. EMDM activity showed the same effect, although 6β-hydroxylase did not show a complete masculinization. Waxman *et al.* (1989), found that testosterone administration from day 35-70, to the non-defeminized male, significantly increased 16α-hydroxylase activity, to 60% of intact male levels.

5.2.4. Conclusions: We demonstrated peripubertal defeminization in males who had neonatal defeminization blocked by ATD administration. Other laboratories demonstrated a peripubertal masculinization phenomenon in the non-neonatally-defeminized (neonatally castrated) male (Dannan *et al.*, 1986; Shimada *et al.*, 1987; Virgo, 1991; Bandiera and Dworschak, 1992). Until more data are presented which *unequivocally* reveal a permanent component to the exogenous peripubertal masculinization phenomenon, the differences seen in our study must be attributed to either ***(a)*** our method of inhibiting defeminization (ATD administration), or ***(b)*** our method of inducing peripubertal imprinting (intact testes).

Proximate peripubertal masculinization steroid: All laboratories that demonstrated the peripubertal masculinization phenomenon, utilized **testosterone propionate**, administered either in a subcutaneous Silastic™ capsule (Dannan *et al.*, 1986; Waxman *et al.*, 1989; Virgo, 1991), or *via* subcutaneous injection (Shimada *et al.*, 1987; Bandiera and Dworschak, 1992). We relied on the presence of the testes

to effect peripubertal masculinization, therefore it can be *suggested* that testosterone, is the proximate peripubertal masculinization steroid. On the other hand, the data indicating that the *ATD/intact* group = *ATD/55 T = intact males*, may indicate that testosterone is the sole testicular factor involved in effecting masculinization and/or maleness, but cannot be *concluded* to be the proximate peripubertal *defeminization* steroid.

Bandiera and Dworschak (1992) found that although testosterone propionate administered to the non-defeminized male, during both neonatal and peripubertal (day 35-49) time frames, could effect a significant increase in 2C11 protein levels, *estrogen* administered during the same time frames, did *not* evoke an increase. Dannan *et al.* (1986), presented similar results, peripubertal (35-70) testosterone administered to the non-defeminized male, effected a *complete* restoration of adult levels of 2C11 and 3A2 protein (and their respective 16α- and 6β-hydroxylations), but estrogen treatment during this same time frame did *not* evoke any increase. Thus, estrogen administered to the *castrated* male does not appear to effect peripubertal defeminization, however, estrogen administration to the *intact* male appears to have different effects. Because we effected peripubertal defeminization by using the intact testes, we must consider the possibility of hormonal interactions, which may affect the process of peripubertal defeminization.

Hormonal interactions in the intact animal: In the intact male, neonatal *or* pubertal administration of estrogens actually *prevented* the adult expression of 2C11 levels (Bandiera and Dworschak, 1992). It is also interesting that

neonatal testosterone treatment, or both neonatal and pubertal testosterone treatment, given to the intact male, significantly *decreased* adult levels of 2C11 (Bandiera and Dworschak, 1992). These results may reflect the adult regulation of 2C11 by the masculine GH pattern, which can be regulated by estradiol (which increases baseline levels; Carlsson *et al.*, 1987), subsequently decreasing 2C11 activity. However, the question arises, *if estrogen is the proximate neonatal imprinting hormone, why would estrogen administration to the* ***intact*** *male* ***prevent*** *neonatal defeminization?*

Literature regarding peripubertal defeminization in the female rat, may assist in determining the mechanism behind endogenous peripubertal defeminization, as data from these studies demonstrate ***(a)*** a permanent defeminization of P-450s, and ***(b)*** a significant interaction between estrogens and androgens. For example, Pak *et al.* (1984) and Pak *et al.* (1985), showed that in the female, the cytochrome P-450-dependent aryl hydrocarbon hydroxylase and 5-reductase, were *not* inducible by testosterone administration in adulthood. They found that when testosterone was administered during the peripubertal period (day 35-50), this resulted in a significant *increase* in testosterone sensitivity of these enzymes in adulthood. Interestingly, in the absence of ovary-derived estrogens (ovariectomy), the same treatment resulted in an even larger sensitivity (increase) to testosterone in adulthood. These results may indicate that during puberty, testosterone is capable of defeminizing the response of hepatic enzymes to subsequent testosterone exposure.

The difference in responsiveness in the ovariectomized *vs.* intact females, may indicate that estrogens can interfere with (but not negate) this defeminization

process. Chang *et al.* (1996) found that peripubertal testosterone administration (day 35-49) to the intact female, did not increase basal activity of 2α-hydroxylase, however combined peripubertal and adult (69-77) testosterone treatment resulted in a significant increase in enzyme activity (sensitization). Preventing the action of estrogen through the estrogen receptor (by prepubertal tamoxifen treatment), enhanced the sensitization of 2α-hydroxylase to subsequent (adult) testosterone treatment, and also revealed a permanent defeminization (increase) of basal 2α-hydroxylase activity.

It is apparent from the above data obtained from the female, that estrogens may interfere with peripubertal defeminization of microsomal enzymes. The presence of estrogens limit the peripubertal sensitization of these enzymes to subsequent testosterone treatment, and the complete absence of estrogens (ovariectomy) results in a greater sensitization (Pak *et al.*, 1984; Pak *et al.*, 1985).

This phenomenon may also be true in the case of *male-specific* P-450s. Chang and Bellward (1996) reported that peripubertal testosterone treatment (day 35-49) of the ovariectomized female rat, resulted in a significant increase in 2α-hydroxylase activity (permanent), and subsequent testosterone exposure (day 81-89) completely masculinized activity of this enzyme. Cadario *et al.* (1992) also found that pubertal testosterone treatment (day 35-49) sensitized 6β-hydroxylase activity, to subsequent adult testosterone treatment, in the ovariectomized female. Imamura *et al.* (1994) found that, in the ovariectomized female rat, the male-specific acetohexamide reductase (not a P-450) could also be sensitized to subsequent (adult)

testosterone exposure, when treated with testosterone during puberty (5-6 weeks of age).

5.3. Why puberty?

5.3.1. Physiological significance: Perhaps it is possible that the massive increase in serum testosterone concentrations during puberty (Dohler and Wuttke, 1975), effects a significant increase in testosterone hydroxylase activities in order to *protect* the male from the excessive amounts of this hormone by increasing testosterone metabolism. In other words, neonatal and/or peripubertal defeminization(s) of testosterone hydroxylases are necessary to ensure higher rates of androgen (and possibly estrogen) elimination in the male, whether the testes are present or not. Adrenal gland derived testosterone increases significantly peripubertally in the absence of the testes, making testosterone metabolism necessary in the castrate as well as the intact male.

Testosterone may directly influence P-450 activity, as Kato and Onoda (1970) demonstrated the ability of androgens to increase the capacity of P-450s to interact with substrates, and that estrogens block this effect. Denef and DeMoor (1972) demonstrated that administration of an androgen receptor antagonist (cyproterone acetate) from birth until day 6, counteracted the masculinization of cortisol metabolism by exogenous testosterone administration. Therefore, it seems reasonable to consider a direct influence of testosterone on P-450, especially during a

time-frame (puberty) in which adult regulatory mechanisms (*i.e.* growth hormone secretory patterns) are only emerging (Eden, 1979).

Regardless of *why* this phenomenon occurs, (which we cannot answer from this study), what is the significance of the peripubertal period? It appears that the normal development of the hormone receptor relies heavily on the ***(a)*** *availability* of the proper hormone, ***(b)*** the proper *time-frame* for imprinting. If peripubertal defeminization is dependent on a hormone/receptor interaction, perhaps there are only two time-frames in which receptors are available to be permanently defeminized: the neonatal time-frame, and the peripubertal time-frame.

5.3.2. Similarities between the neonatal and peripubertal time-frames: Any attempt to explain why imprinting can occur during puberty, begins with an examination of the physiological state of the male rat, during this time-frame. Perhaps there is a ***repetition of a specific hormonal environment***, unique to the neonatal and pubertal time periods, which allows for the imprinting "mechanism" to occur. This hypothesis is only plausible if the *imprinting mechanism* (*i.e.* estrogen through the estrogen receptor) is identical to that in the neonatal time-frame.

Sex hormone levels: Male rats have high serum *estradiol* concentrations from day 21 of gestation, to approximately postnatal day 1 (Rhoda *et al.*, 1984), and there appears to be a transient increase in serum estradiol concentration between day 9-23, after which time, levels drop significantly (Dohler and Wuttke, 1975). Estrogen receptors are abundant in a number of areas of the

brain, appearing on day 19 of gestation (Pasterkamp *et al.*, 1996), correlating with the high serum estradiol concentrations. Serum estradiol concentrations during puberty are very low (Dohler and Wuttke, 1975).

Serum *testosterone* levels, on the other hand, are as high in the first 19 days of life as those of the adult, however the levels obtained during puberty are much greater than during either time-frame (neonatal or adult), rising from day 35, and peaking around day 50-55 (Dohler and Wuttke, 1975). There also is a reported testosterone/estradiol "surge" during the first 2 hours of life in the male rat (Rhoda *et al.*, 1984). Serum and hypothalamic DHT levels are undetectable in the neonate (Rhoda *et al.*, 1984), however, pituitary DHT formation was actually *higher* in the neonate, than in the adult (Denef *et al.*, 1974). Serum DHT has been shown to peak in the male around day 56 (Corpechot *et al.*, 1981).

Although the neonatal and peripubertal endocrinology is not the same, perhaps the high levels of DHT, coupled with the low levels of estradiol (opposite to that of the neonate), induce brain aromatase activity during puberty (*i.e.* the mechanism of defeminization is the same). These two hormones can synergistically induce aromatase activity (Roselli, 1991), and could possibly increase the local conversion of testosterone to estradiol. Theoretically, this combination of events could effect defeminization, providing peripubertal defeminization involves a similar mechanism to neonatal defeminization.

It is apparent that although neonatal defeminization of EMDM involves a significant increase in uninduced enzyme activity, peripubertal masculinization

effects a significantly larger 2.4-fold increase in specific activity. This suggests that either *(a)* neonatal defeminization and peripubertal defeminization are brought about by different mechanisms, or *(b)* the *same mechanism evokes a slightly different response in terms of uninduced specific activity.* The hydroxylations of testosterone show slightly different profiles, as leaving the testes *in situ* until day 70, resulted in basal activities of all three isozymes, that were significantly less than those of the day 55 castrates. This 'trend' seems to support the concept that the peripubertal defeminization process is very dynamic throughout day 35-70, i.e. *the response of the enzyme system to a constant stimulus varies throughout puberty.*

5.3.3. <u>The dynamics of puberty</u>: Specific hormonal treatments may have differing effects, depending on the time-frame during which they are administered, in other words, there may exist *two* windows of opportunity for sex hormones to be exposed to the pertinent target tissues, and ultimately exert their defeminizing effects. One window in the neonatal time-frame, and one in the peripubertal time-frame. Even if the imprinting mechanism does not involve the defeminization of specific brain characteristics, the following characteristics of the pubertal time-frame serve to explain the unique aspects of this period, which may render *another* imprinting mechanism available.

<u>Sensitivity from birth through puberty</u>: The sensitivity of the hypothalamo-pituitary axis, during different time-frames in the life of a rat, has been thoroughly investigated. It has been demonstrated that the administration of

morphine and/or its antagonist (naloxone), have differing effects on serum LH and prolactin levels in prepubertal males, depending on the *age* of the animal (Ieiri *et al.*, 1979). Studies on the pituitary regulation of (insulin-like-growth-factor) IGF-I, IGF-II, and IGF-binding protein by GH/TSH, show that the juvenile rats required the intact pituitary for somatic growth, more so than the infant (Glasscock *et al.*, 1991), suggesting that the infants may be more sensitive to available hormones. This is consistent with data from Bloch *et al.* (1974), Ojeda and Ramirez (1973/74), and Negro-Vilar *et al.* (1973b), who suggest that the sensitivity of the hypothalamo-pituitary-gonadal axis to sex hormone feedback, is *greatest* in the neonate, *intermediate* in the adult, and *least* in the pubertal rat. This demonstrates how a similar hormonal treatment, may evoke different quantitative responses, depending on the time of hormone administration.

5.4. Possible target sites

5.4.1. <u>The brain</u>: An example of a tissue developing at different intervals within the pubertal time-frame, is the brain. Several regions are not permanently differentiated prior to puberty, and are still able to be manipulated by sex steroids during puberty. For example, the anteroventral periventricular nucleus (AVPv) volume develops between day 30-40, and the length becomes sexually differentiated between days 60-80 (Davis *et al.*, 1996). Bloch and Mills (1994) also demonstrated that the peripubertal testosterone treatment of the non-defeminized male, effectively defeminized the size of several sexually dimorphic components of the medial

preoptic area. Peripubertal (day 15-30) testosterone treatment decreased the width of the periventricular preoptic area, and also decreased the volume of the anteroventral periventricular nucleus.

Certain aspects of male rat reproductive behaviour and endocrinology, can also be defeminized peripubertally in the absence of neonatal defeminization. Bloch and Mills (1995) found that administration of testosterone from day 15-30, to the non-defeminized male, resulted in *reduced* lordosis and proceptive behaviours, *increased* mounting and intromission behaviours, and also *reduced* plasma LH and FSH surges (*i.e.* feminization). Primus and Kellogg (1990) also found that the pubertal secretion of androgens are necessary for the activation of environmental-related social interaction, in the male rat.

In certain areas of the brain, estrogen receptor occupation (Yuan *et al.*, 1995), and content (Rainbow *et al.*, 1982), are decreased upon castration in adulthood. In addition, testosterone has been shown to down-regulate estrogen-receptor mRNA hybridization in specific regions of the male rat brain (Simerly and Young, 1991), and to restore luteinizing-hormone-releasing-hormone (LHRH) content in the medial basal hypothalamus (MBH) following a castration-induced decrease (Kalra and Kalra, 1980). These results demonstrate that testosterone can still act on neural substrates well beyond the neonatal period to defeminize and masculinize endocrine and behavioural function in the male rat. **Table 5.4.1.** demonstrates the areas of the brain responsive to hormonal manipulation, their time-frame of development, as well as the suggested "critical period" for development.

Table 5.4.1. Suggested critical periods for neonatal defeminization of some sexually dimorphic brain areas, and respective permanence in adulthood.

Area	**Critical period**	**Plasticity**
medial preoptic nucleus MPN (including SDN)	defeminized by day 2 [1]	adult castration does not affect size [2]
preoptic area POAH	synaptic density plateaus by day 20-30 [3] and volume imprinted by 14 [4]	adult castration has no effect on T or E receptor concentration [5] or size [4]
ventromedial nucleus VMN	not clear	E in adulthood can increase the number of synapses [6]
arcuate nucleus ARN	completion of synaptogenesis is delayed to the onset of puberty [7,8,9]	E can facilitate synaptogenesis peripubertally and in adulthood [10,11]
suprachiasmatic nucleus SCN	synapse density reaches adult level before day 21 [12]	not clear
medial amygdaloid nucleus MAN	synapse density reaches adult level before day 21 [13]	not clear
periventricular nucleus PVN	volume (AVPv) develops between day 30-91 [14]	not clear
medial basal hypothalamus MBH	E and T receptor levels imprinted by day 7 [5]	adult castration fails to change E or T receptor levels [5]

Note: For numbered references, see **Appendix D.**.

5.4.2. <u>The sensitive hormone receptor</u>: Because receptors play a large role in the neonatal defeminization process (Reyes and Virgo, 1988), perhaps they also play a role in peripubertal defeminization. Csaba and Inczefi-Gonda (1992) suggest that in general, the quality of hormone receptors is genetically encoded, and the receptor number and affinity characteristics become established during maturation. A *depletion* or *excess* of adequate hormone or influence of *inadequate* hormone (which can bind to the receptor) leads to abnormal receptor development. This may help explain why neonatal testosterone administered to the intact male significantly decreased adult levels of 2C11 (Bandiera and Dworschak, 1992). Perhaps the excess amount of testosterone damaged its own receptor, and subsequently prevented a normal feedback mechanism from occurring.

Estrogen administered to the intact newborn male rat, has been shown to alter sexual development (delay both the increase in testosterone levels at puberty, and also the pubertal increase in the weight of the testes and seminal vesicles) (Brown-Grant *et al.*, 1975). It has also been shown to alter the developmental pattern of *androgen* receptor expression, in the rat prostate (Prins and Birch, 1995). Csaba and Inczefi-Gonda (1992) found that a single dose to 17β-estradiol or progesterone, within 24 hours after birth, led to a significant decrease in uterine estrogen-receptor binding capacity. Pap and Csaba (1995) found that a single perinatal allylestrenol treatment on its own, resulted in a doubling of serum testosterone concentration in adulthood, whereas allylestrenol in adulthood (beyond the critical period) had no

effect. Therefore, although the receptor may be present, the receptor affinity for the hormone, or the hormone feedback mechanism, may be permanently altered.

In addition to data from Bandiera and Dworschak (1992) (above), exposure *in utero*, or within a few days of birth, to estrogenic or androgenic compounds has been shown to result in alterations in hepatic metabolism in adult animals. Neonatal treatment with diethylstilbestrol (DES), resulted in decreased 3A2 levels in adulthood (Zangar, 1993), and the weak estrogenic agent, clomiphene citrate, administered on day 3 of life to the male, resulted in a significant reduction in 16α-hydroxylase activity (active on dihydroepiandrosterone) in adulthood (Tabei and Heinrichs, 1976).

5.4.3. <u>The liver</u>: As mentioned earlier, many unique physiological events occur during the peripubertal time-frame, which do not occur at any other time in the life of the rat. One such event is a massive growth spurt of various organs, including the liver (MacLeod *et al.*, 1972). Because these cells are dividing at such an enormous rate, and because the cell progeny are 'new' cells, perhaps these *unimprinted* new cells require a time-frame in which they, too, become imprinted with respect to specific hormones and receptors. Thus, the question arises, "*how are the **newly** formed cells imprinted to **respond** to adult regulatory mechanisms?*".

It has been demonstrated that during liver regeneration (*i.e.* liver growth), estrogen treatment had a long-lasting effect on the inducibility by phenobarbital of the hepatic microsomal system of the female, but not the male (Csaba *et al.*, 1987). They suggest that availability for imprinting depends on the *developmental state* of

the target cell. Therefore, perhaps the new liver cells are *responsive* to hormonal imprinting during this pubertal growth spurt. It has also been demonstrated that androgens and low doses of estrogens affect three hepatic androgen-dependent microsomal enzyme activities (3α- and 3β-hydroxysteroid dehydrogenase and 5α-reductase) by acting at different levels of central regulation, whereas large doses of estrogens act *directly* on the liver *via* hepatic estrogen receptors (Lax *et al.*, 1983).

5.5. Possible contributing factors

5.5.1. <u>P-450 protein levels</u>: The following researchers demonstrated that the change in P-450 specific activity following hormonal manipulation, is not likely due to a change in P-450 content. Kramer *et al.* (1979), demonstrated that adult castration did not have any effect on microsomal protein content or cytochrome P-450 concentration, and Virgo (1985) showed that somatostatin or testosterone therapy to the adult castrate did not affect P-450 content. On the other hand, Al-Turk *et al.* (1981) presented data indicating that tamoxifen treatment (an estrogen receptor blocker), reduced plasma testosterone levels, and subsequently decreased microsomal P-450 content.

5.5.2. <u>P-450 cofactor availability</u>: The decrease in EMDM activity following adult castration (El Defrawy El Masry *et al.*, 1974), may be *partially* attributed to a slight decrease in cytochrome *c* reductase activity, indicating that if a

necessary cofactor in the P-450 enzyme system is perturbed, this could affect the specific activity of that particular system. Virgo (1985 and 1991) also demonstrated that testosterone and/or somatostatin therapy can significantly increase cytochrome *c* reductase activity, and corresponding EMDM activity, in either the neonatal or adult castrate. Waxman *et al.* (1989) also demonstrated that hypophysectomy reduced NADPH P-450 reductase activity by 63-77%, which they suggested to contribute to their observed changes in P-450 activity. It has been determined that cytochrome *c* reductase is the rate-limiting component in the P-450 system (Miwa *et al.*, 1978), but it is unlikely that the *significant* changes in enzyme specific activities are strictly due to these *slight* changes in cofactor availability. Our work was performed in vitro, with constant concentrations of microsomal protein and NADPH. Therefore, any change in specific activity of the enzyme system cannot be attributed to changes in these parameters. We can only suggest increasing/decreasing *amounts/activities* of isozyme or of cytochrome *c* reductase.

5.5.3. <u>Other hormones</u>: It is interesting that monosodium glutamate (MSG)-treated neonatal males which had an almost complete loss of circulating GH in adulthood, had a significantly *decreased* 6β-hydroxylase activity as adults (Waxman *et al.*, 1995). This suggests that there may be another pituitary-dependent hormone, which regulates this reaction, as complete removal of GH (by removing the pituitary gland), significantly *increased* 6β-hydroxylase activity (Yamazoe *et al.*, 1986b; Kato *et al.*, 1986). Waxman *et al.* (1995) indicated that neonatal MSG treatment did *not*

result in a loss of *IGF-1* (insulin-like growth factor), whereas Mathews *et al.* (1989) found that hypophysectomy significantly *decreased* IGF-I mRNA expression. Many effects of growth hormone may be indirectly mediated via this hormone (Zapf *et al.*, 1984).

It is possible that glucocorticoids play a role in regulation of 6β-hydroxylase, as dexamethosone treatment of male hepatocytes (*in vitro*) increased activity of this isozyme, and GH reversed the stimulation (Vind *et al.*, 1992). Waxman *et al.* (1990) found that a more *complete* suppression of 3A2 and 6β-hydroxylase activity by GH in the hypophysectomized male, was effected when thyroxine (T_4) was concomitantly administered. *In vitro* studies also indicated that dexamethosone *increased* 2C11 mRNA expression in hepatocytes (as well as *in vivo*), but concomitant dexamethosone and growth hormone exposure decreased 2C11 mRNA (Liddle *et al.*, 1992). They suggest that 2C11 mRNA expression is under the primary GH regulation. The same study also demonstrated that L-triiodothyronine (T_3) exposure also decreased 2C11 mRNA expression to 46% of control. The growth hormone regulation of 15β-hydroxylase, has also been shown to be potentiated by concomitant administration of T_4 and cortisol in the female rat (Mode *et al.*, 1989a).

Because growth hormone plays such an important role in the regulation of 2C11 and 3A2, perhaps GH is also involved in the imprinting process, either neonatally, or during puberty. Gabriel *et al.* (1989) found that serum GH increased (8-fold) from day 25-45 in the rat, and again (5-fold) by day 90, and Eden (1979) indicated that the sexual dimorphism in secretory pattern becomes apparent during

this period. Somatostatin content in the hypothalamus has also been shown to increase significantly from day 25-95, and between day 25-35 in the median eminence (Gabriel *et al.*, 1989).

Hepatic GH receptor mRNA (Mathews *et al.*, 1989) and hepatic GH binding (Maes *et al.*, 1983), are minimally expressed at birth, and rise to adult levels by day 35-40 (peripubertally). Tiong and Herington (1992) report that no measurable GH binding activity occurred before day 20. Mathews *et al.* (1989) found that hypophysectomy and GH treatment did not affect hepatic GH receptor mRNA levels. Serum growth hormone binding protein (GHBP) activity was detectable at 10 days of age and rose to adult levels by day 50 (Tiong and Herington, 1992). Because no hepatic GH binding activity was noted before day 20 (Tiong and Herington, 1992), and hepatic GH receptors are probably not sexually dimorphic (changes in GH secretory patterns can modulate P-450s), this probably excludes any contribution of GH to the *neonatal* defeminization process. However, this does not exclude GH from the peripubertal defeminization process.

5.5.4. <u>Special form of adolescent imprinting</u>: Perhaps there exists a *special form* of hormonal imprinting that occurs only during adolescence. In the female rat, it has been shown that nandrolone (an anabolic steroid) treatment at day 42 and 49, results in a significant decrease in the density of thymic glucocorticoid and uterus soluble estrogen receptors (Csaba and Inczefi-Gonda, 1993). The same laboratory also found that gonadotrophin hormone treatment (FSH and LH) on day

49, resulted in a significant reduction of thyroxine in adulthood, and a decrease in thyroidic response to TSH exposure (Csaba and Nagy, 1990). It is also interesting that neonatal exposure to dexamethasone resulted in a decrease in thymic glucocorticoid receptors at 56 days, and exposure at 28 days had similar effects (Csaba and Inczefi-Gonda, 1990).

5.6. Summary

In this study, we utilized two unique techniques, in order to block the neonatal defeminization of 3A2 and 2C11, and subsequently effect a "peripubertal masculinization" of these enzymes. We utilized an aromatase inhibitor (ATD), to block the estrogen-induced defeminization of both P-450s, and left the testes *in situ* throughout the peripubertal period to effect peripubertal masculinization.

1. We demonstrated that males exhibit higher velocities of ethylmorphine demethylase, 6β-hydroxylase, 2α-hydroxylase, 16α-hydroxylase, and aniline hydroxylase, when compared to the female. This is in accordance with data from other laboratories, although aniline hydroxylase activity is not usually characterized as being sexually differentiated. The 7α-hydroxylase, was also sexually differentiated in our study, which was expected (females > males). We also observed significant sex differences in total cytochrome P-450 levels, as well as in cytochrome *c* reductase activity (males > females). The slight differences in P-450 levels and

reductase activity, were not great enough to account for the large sex differences seen in the other enzyme activities.

2. In our laboratory, neonatal defeminization is characterized as an increase in basal activities, to velocities greater than those of the female. The EMDM, 2α-hydroxylase, and 16α-hydroxylase behaved thus. The data on the 6β-hydroxylase, did not *statistically* reveal defeminization as an increase in activity over that of the female, although the activity was 2-3 fold greater in the defeminized males.

3. Neonatal ATD administration (day 0-21) prevented defeminization (as characterized as an increase in basal enzyme activity), of EMDM, 6β-hydroxylase, 2α-hydroxylase, and 16α-hydroxylase. The method of delivery of this compound (subcutaneous Silastic™ capsule), as well as the time-frame for delivery (day 0-21), were demonstrated as being sufficient to block defeminization.

4. Neonatal ATD administration prevented an increase (feminization) in basal 7α-hydroxylase activity, but did not prevent testosterone suppression in adulthood. Blocking estrogen production was not effective in preventing testosterone-induced suppression of this enzyme in adulthood.

5. Adult masculinization (preceded by neonatal defeminization) by the testes, accounts for 31% of EMDM activity, 46% of 6β-hydroxylase activity, 40% of 2α-hydroxylase activity, and 51% of 16α-hydroxylase activity.

6. In the non-neonatally-defeminized male, EMDM, 6β-hydroxylase, 2α-hydroxylase, and 16α-hydroxylase, are responsive to testosterone in adulthood, and we suggest the term **"maleness"** to identify this response.

7. In the non-neonatally-defeminized male, peripubertal testicular secretions are sufficient to completely *masculinize* (implies peripubertal defeminization) EMDM, 6β-hydroxylase, 2α-hydroxylase, and 16α-hydroxylase. This occurs if the testes remain *in situ*, until sacrifice.

8. In the absence of neonatal defeminization, peripubertal testicular secretions are effective at defeminizing (permanently increasing for at least 15-30 days) basal EMDM activity, which remained greater than the female whether castrated on day 55 or day 70.

9. In the absence of neonatal defeminization, peripubertal testicular secretions are effective at defeminizing (permanently increasing for at least 15 days) basal 6β-, 2α-, and 16α-hydroxylase activities, when the testes remain *in situ* until day 55. Castration at day 55 revealed that basal enzyme activities that were significantly greater at this time, than at any other time (*i.e.* after castration at 21, 55, or 70 days). Castration on day 55 also revealed a significant increase in 7α-hydroxylase activity, which was not apparent in the day 70 or day 21 castrate.

10. The peripubertal defeminization process appears to be completed by day 55, as basal EMDM activities were not *further* increased when the testes remain *in situ* until day 70. In addition, leaving the testes *in situ* until day 70, appeared to *reverse* the defeminization of 6β-, 2α-, and 16α-hydroxylases, which was evident on day 55.

APPENDIX A

Figure A.1. An example of a standard curve of the Bradford Protein Assay (microassay procedure) (Bradford, 1976), showing standard deviations and 95% confidence interval. The assay was run eight times to demonstrate both precision and accuracy. For description of the assay, see section **3.5.1.**.

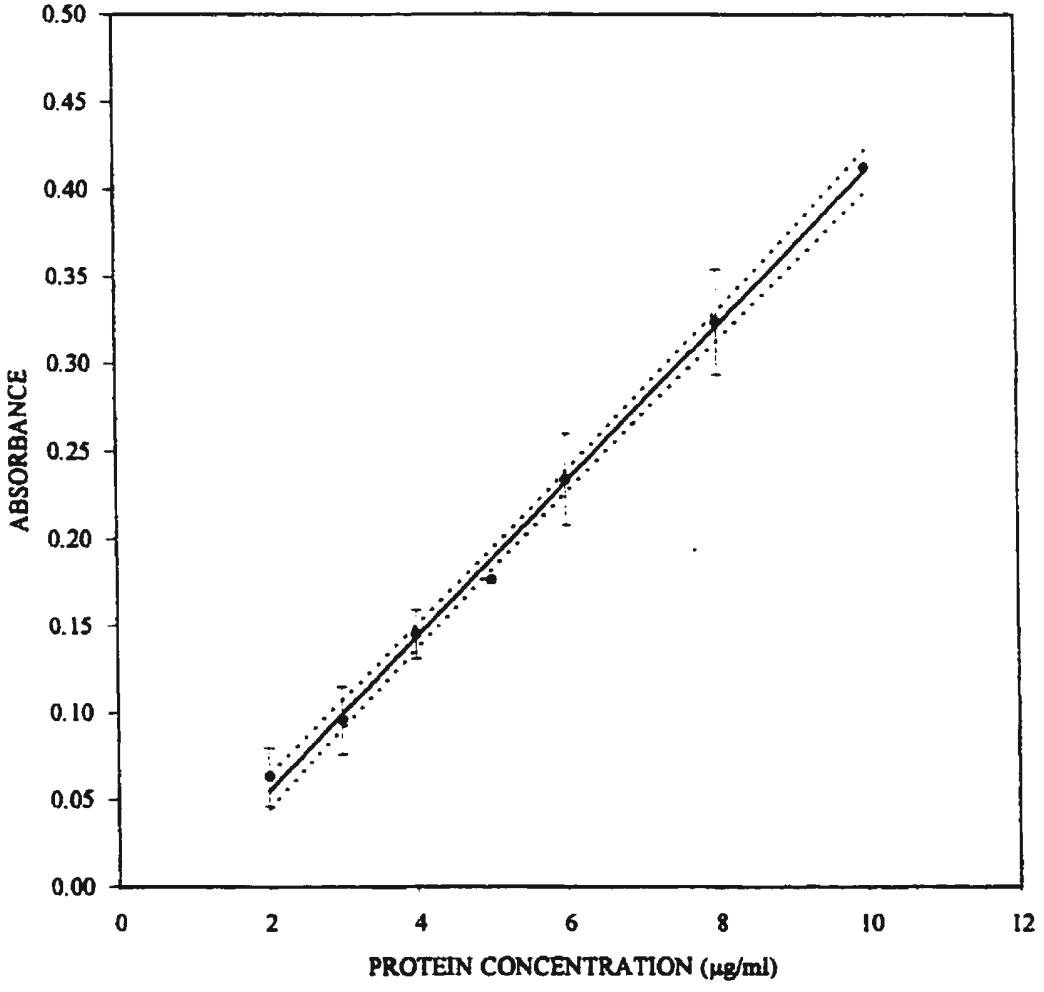


Figure A.2. Formaldehyde standard curve with 95% confidence intervals, for use in the ethylmorphine demethylase assay. For description of the assay, see section 3.5.2..

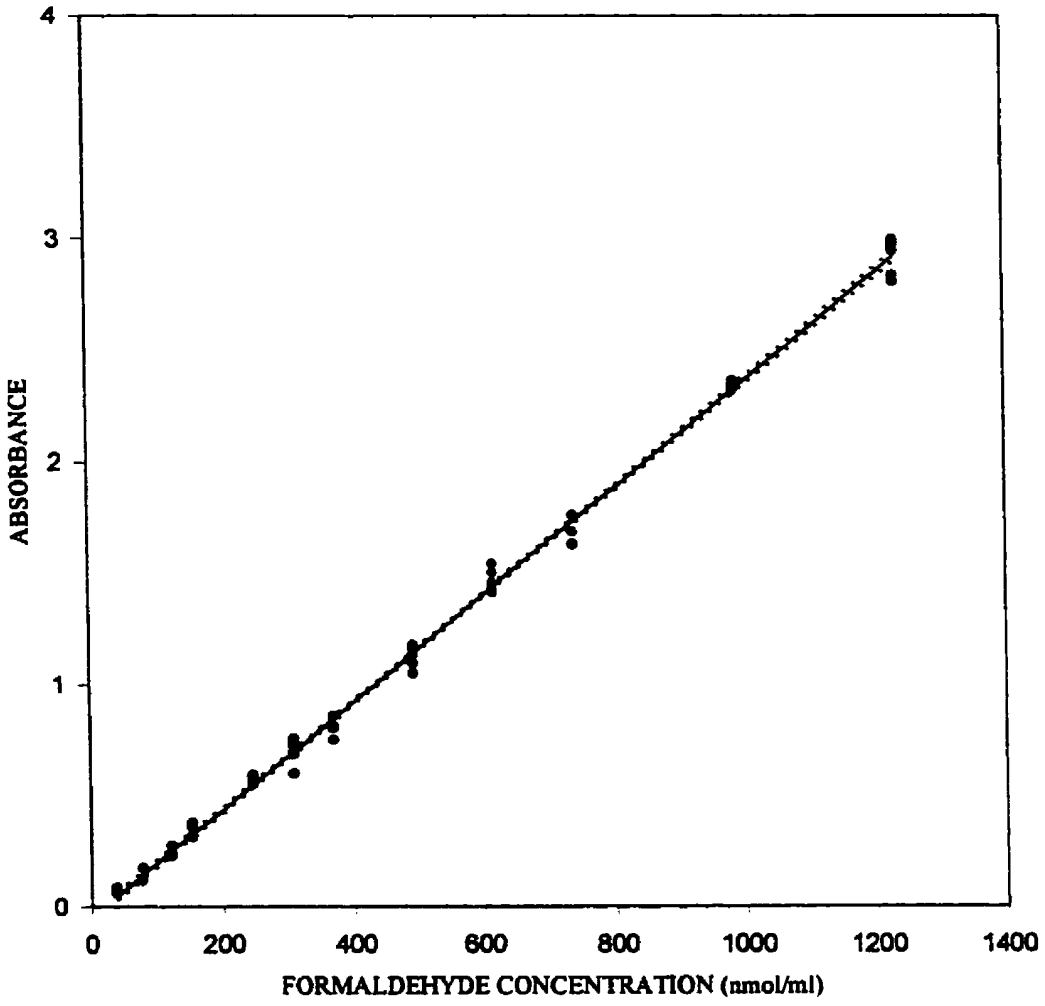


Figure A.3. Hydroxyaniline (4-aminophenol) standard curve showing standard deviations and 95% confidence intervals. The assay was repeated six times, on different days, to demonstrate both precision and accuracy. For description of the assay, see section 3.5.5..

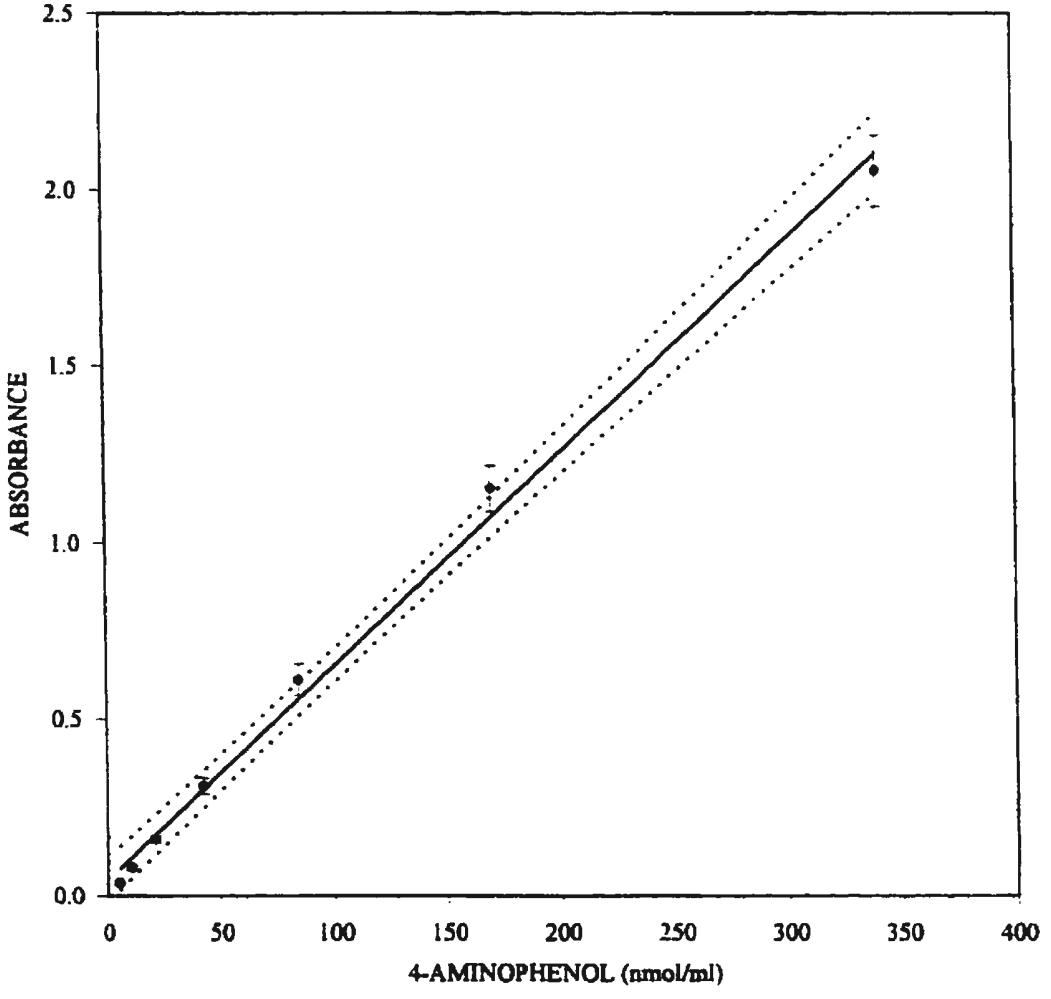


Figure A.4. A standard curve of the 16α-hydroxylation of testosterone, showing standard deviations and 95% confidence intervals. The assay was repeated three times, on different days, to demonstrate both precision and accuracy. For description of the assay, see section **3.5.6.**.

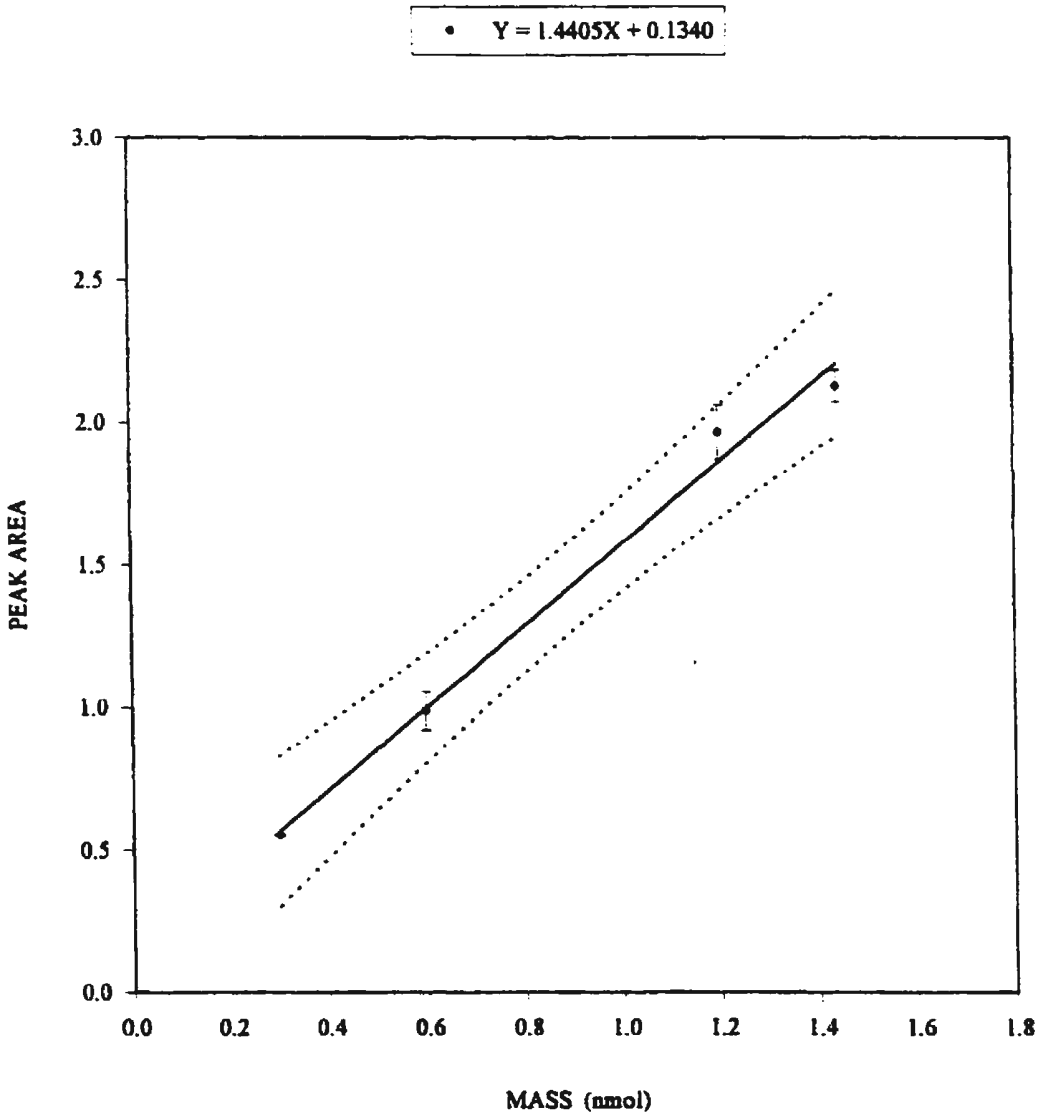


Figure A.5. A standard curve of the 2α-hydroxylation of testosterone, showing standard deviations and 95% confidence intervals. The assay was repeated three times, on different days, to demonstrate both precision and accuracy. For description of the assay, see section **3.5.6.**.

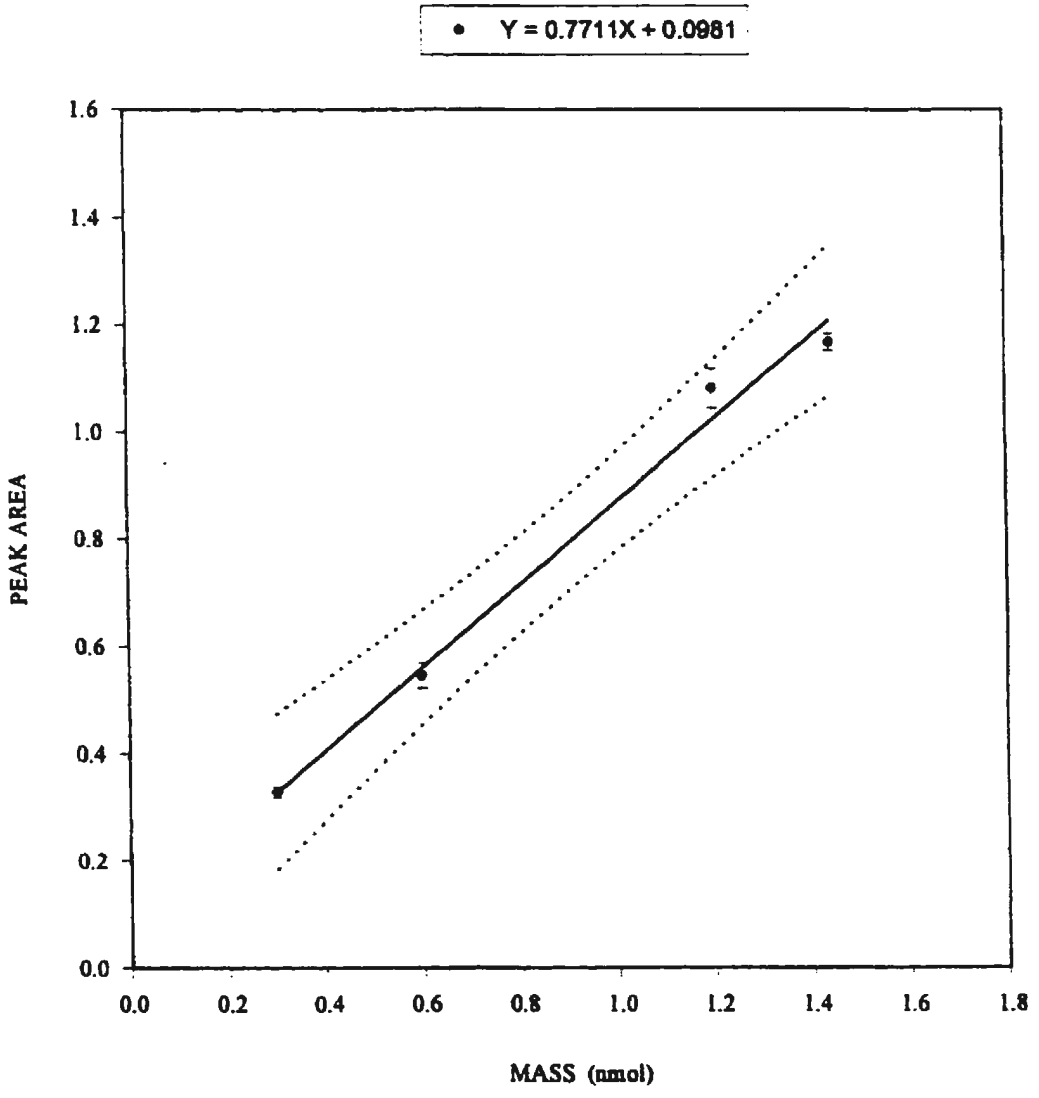


Figure A.6. A standard curve of the 6β-hydroxylation of testosterone, showing standard deviations and 95% confidence intervals. The assay was repeated three times, on different days, to demonstrate both precision and accuracy. For description of assay, see section **3.5.6.**.

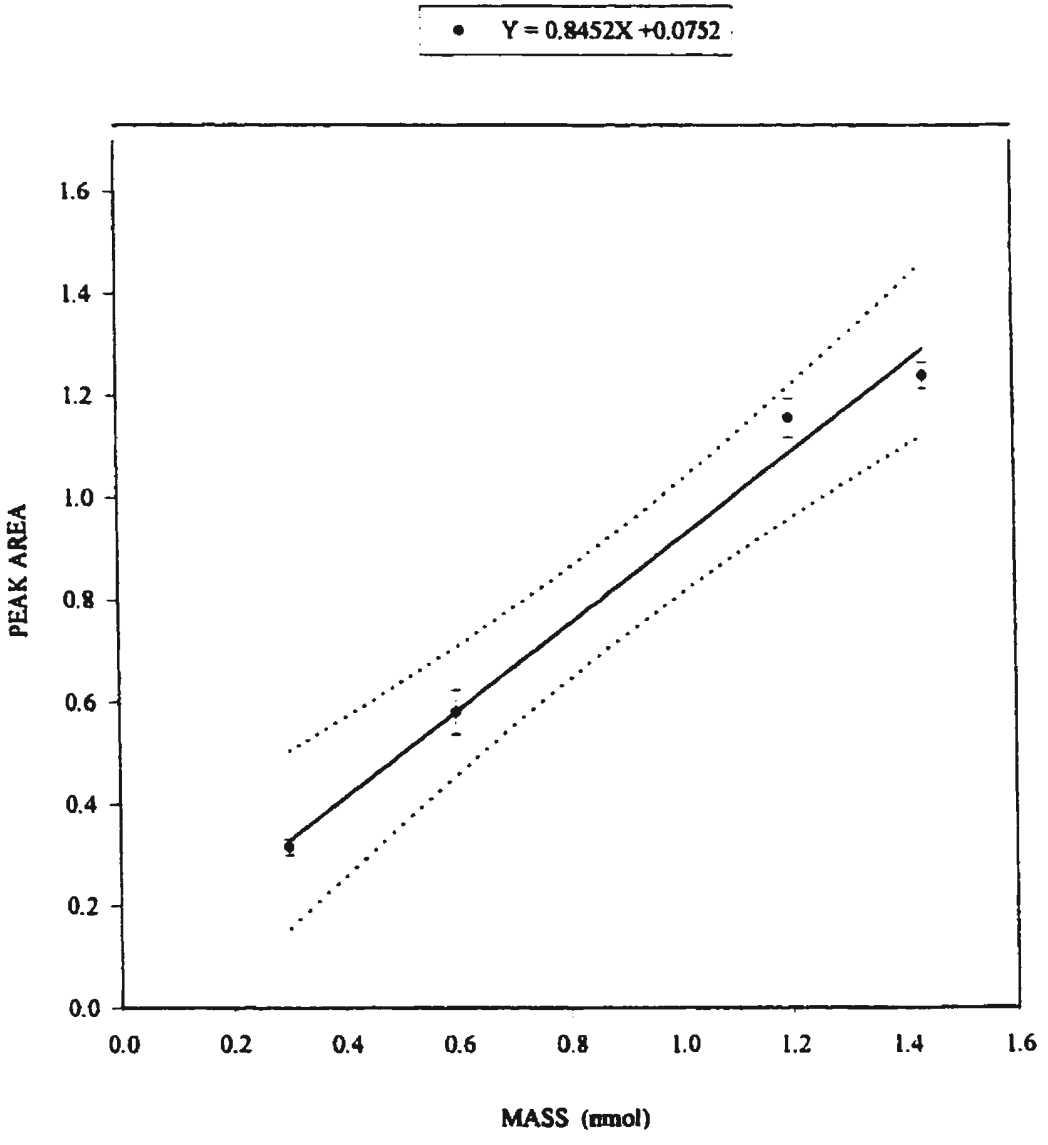
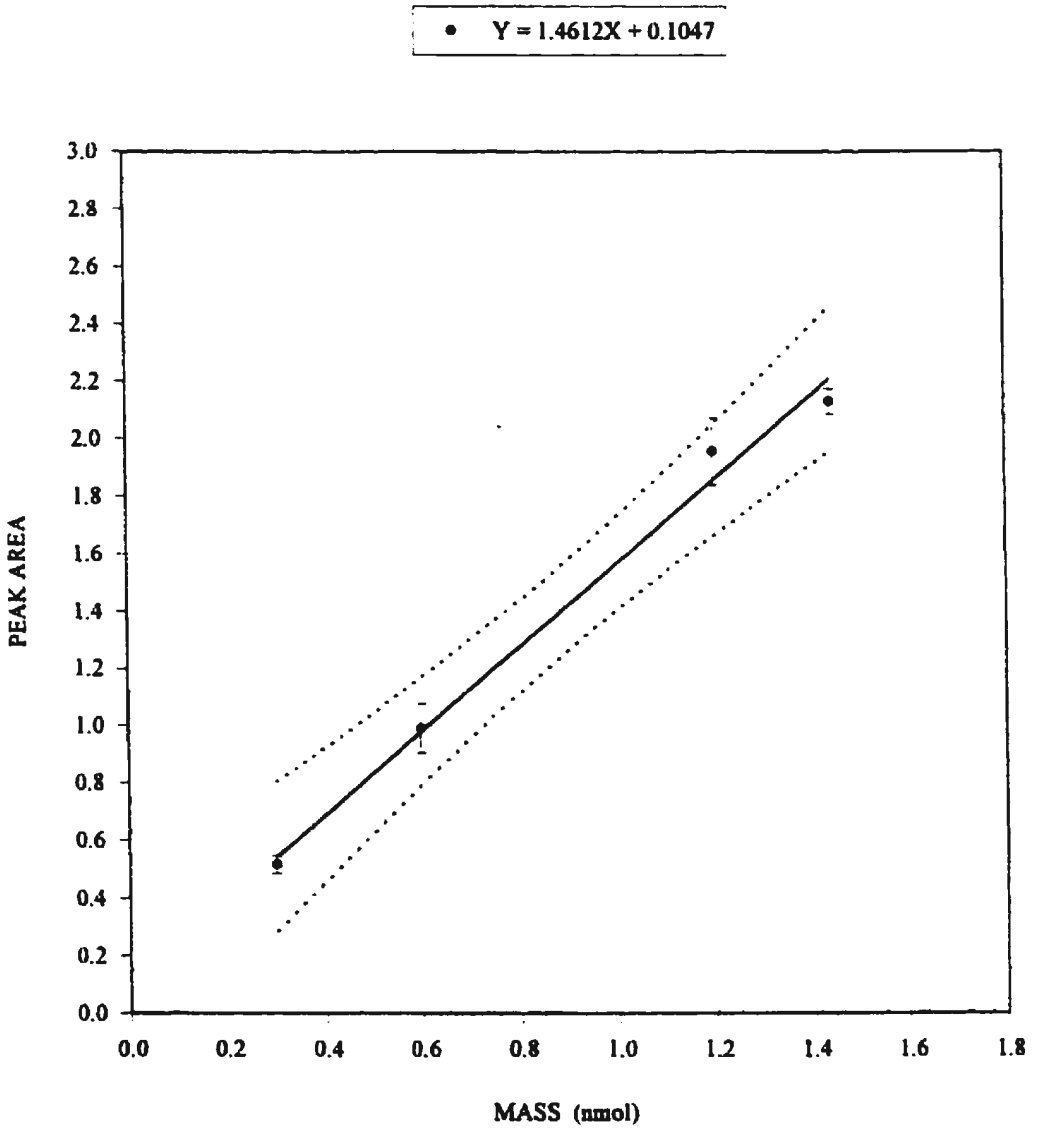


Figure A.7. A standard curve of the 7α-hydroxylation of testosterone, showing standard deviations and 95% confidence intervals. The assay was repeated three times, on different days, to demonstrate both precision and accuracy. For description of the assay, see section **3.5.6.**.



APPENDIX B: P-450 Nomenclatures (names given by other laboratories).

P-450	Names	Laboratory
2C11	2c	Waxman
	h	Levin
	UT-A	Guengerich
	RLM5	Schenkman
	male	Kato
	PB2a	Wolf
	M1	Omura
	16a	Gustafsson
3A2	2a	Waxman
	PB/PCN-E	Guengerich
	PCNb/c	Halpert
	PB-1	Imaoka
2A1	UT-F	Guengerich
	PB-3	Waxman
	RLM-2b	Schenkman
	a	Levin
2E1	RLM6	Schenkman
	j	Levin

From: Guengerich *et al.* (1982); Kamataki *et al.* (1983); Waxman (1984); Ryan *et al.* (1984a); Wolf (1986); Matsumoto *et al.* (1986); Schenkman *et al.*, 1987; Halpert (1988); Imaoka *et al.* (1988).

APPENDIX C: MATERIALS

CHEMICAL NAME	CHEMICAL FORMULA	SUPPLIER
Acetonitrile		Fisher Scientific Company
Acetyl acetone	$CH_3COCH_2COCH_3$	Fisher Scientific Company
Albumin, bovine	98-99% albumin, 15.8% N	Sigma
4-aminophenol hydrochloride	$C_6H_7NO \bullet HCl$	Sigma
Ammonium acetate	CH_3COONH_4	Fisher Scientific Company
Aniline	C_6H_7N	Sigma
ATD	1,4,6-androstatriene-3,17-dione	Steraloids
Barium hydroxide	$Ba(OH)_2 \bullet 8H_20$	J.T. Baker Chemical Company
Carbon monoxide	CO	Alphagaz, Canadian Liquid Air, Limited
Cytochrome c	from horse heart, 99%	Sigma
Dichloromethane	CH_2Cl_2	Fisher Scientific Company
Ethyl alcohol		Commercial Alcohols, Ltd.
Ethylmorphine hydrochloride		BDH Chemicals
Glacial acetic acid		Baxter Corporation
Glucose-6-phosphate	disodium salt - hydrate analytical grade, 99% pure $C_6H_{11}O_9PNa_2$	Sigma
Glucose-6-phosphate dehydrogenase	from bakers yeast	Sigma
Glycerin (glycerol)	$CH_2OHCHOHCH_2OH$	Fisher Scientific Company
Hydrochloric acid	HCl	Fisher Scientific Company
Magnesium chloride	$MgCl_2$	Mallinkrodt Incorporated

Materials Table (continued)

Methanol	CH_3OH, HPLC grade and analytical grade	Fisher Scientific Company*
NADP **nicotinamide adenine dinucleotide phosphate**	sodium salt analytical grade, 99% $C_{21}H_{27}N_7O_{17}P_3Na$	Sigma
NADPH **β-nicotinamide adenine dinucleotide phosphate**	reduced form, tetrasodium salt, 97% $C_{21}H_{26}N_7O_{17}P_3Na_4$	Sigma
Phenol	C_6H_5OH	Fisher *
Potassium chloride	KCl	Mallinkrodt Incorporated
Potassium cyanide	KCN	Mallinkrodt Incorporated
Potassium phosphate (monobasic)	KH_2PO_4	Mallinkrodt Incorporated
Potassium phosphate (dibasic)	$KH_2HPO_4 \bullet 3H_2O$	Mallinkrodt Incorporated
Semicarbazide hydrochloride	$H_2NCONHNH_2 \bullet HCl$	Aldrich Chemical Company
Sodium carbonate	Na_2CO_3, (anhydrous)	Mallinkrodt Incorporated
Sodium dithionite	$Na_2S_2O_4 \bullet H_2O$	BDH Laboratory Supplies
Sucrose	$C_{12}H_{22}O_{11}$	Mallinkrodt Incorporated
Testosterone	Δ^4-androsten-17β-ol-3-one	Sigma
Trichloroacetic acid	CCl_3COOH	Fisher Scientific Company
Tris(hydroxymethyl)-aminomethane base	$C_4H_{11}NO_3$	Sigma
Water (Optima)	H_2O, HPLC grade	Fisher Scientific Company
Zinc sulfate	$ZnSO_4 \bullet 7H_2O$	Fisher Scientific Company

Appendix C (continued)

CHEMICAL SUPPLIER ADDRESSES

SUPPLIER	ADDRESS
Aldrich Chemical Company	Wisconsin, U.S.A.
Alphagaz, Canadian Liquid Air, Ltd.	Canada
Baxter Corporation	Toronto, Ontario, Canada
BDH Laboratory Supplies	*England; Toronto, Ontario
Commercial Alcohols, Ltd.	
Fisher Scientific Company	*Nepean, Ontario, Canada; Fair Lawn, New Jersey, U.S.A.
J.T. Baker Chemical Company	New Jersey, U.S.A.
Mallinkrodt Incorporated	Paris, Kentucky, U.S.A.
Sigma	St. Louis, Missouri
Steraloids	Newport, Rhode Island

APPENDIX D

Appendix to Table 5.4.1.

1. Jacobsen *et al.*, 1981
2. Gorski *et al.*, 1978
3. Lawrence and Raisman, 1980
4. Dorner and Staudt, 1968
5. Babichev *et al.*, 1990
6. Carrer and Aoki, 1982
7. Matsumoto and Arai, 1976
8. Matsumoto and Arai, 1981
9. Arai and Matsumoto, 1978
10. Matsumoto and Arai, 1977
11. Matsumoto and Arai, 1979
12. LeBlond *et al.*, 1982
13. Nishizuka and Arai, 1981
14. Davis *et al.*, 1996

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